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<p>(21) International Application Number: PCT/US93/08047 (22) International Filing Date: 27 August 1993 (27.08.93) (30) Priority data: 07/936,792 28 August 1992 (28.08.92) US (60) Parent Application or Grant (63) Related by Continuation US 07/936,792 (CIP) Filed on 28 August 1992 (28.08.92) (71) Applicant (for all designated States except US): THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10666 N. Torrey Pines Road, TPC-8, La Jolla, CA 92037 (US).</p>		<p>(72) Inventors; and (75) Inventors/Applicants (for US only) : RUF, Wolfram [DE/ US]; 643 Westbourne Street, La Jolla, CA 92037 (US). MUELLER, Barbara, M. [DE/US]; 3950 Mahaila Ave- nue, #R-34, San Diego, CA 92122 (US). REISFELD, Ralph, A. [US/US]; 8538 Nottingham Place, La Jolla, CA 92037 (US). EDGINGTON, Thomas, S. [US/US]; 2362 Avenida de la Playa, La Jolla, CA 92037 (US). (74) Agents: LOGAN, April, C. et al.; The Scripps Research Institute, 10666 N. Torrey Pines Road, TPC-8, La Jolla, CA 92037 (US). (81) Designated States: AU, CA, FI, JP, NO, US, European pa- tent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.</p>
<p>(54) Title: INHIBITION OF TUMOR METASTASIS VIA NEUTRALIZATION OF TISSUE FACTOR FUNCTION</p> <p>(57) Abstract</p> <p>The present invention relates to methods and compositions for preventing, inhibiting, or treating metastasis, as well as to methods of making and using such compositions. More particularly, the present invention relates to molecules that bind tissue factor (TF) and disrupt the TF.VIIa complex, thereby inhibiting the onset and progression of metastasis. In addition, the present invention relates to molecules capable of disrupting tissue factor function, or the function of TF.VIIa complexes. The present invention also relates to methods and compositions useful in disrupting or modulating cell-surface TF expression, including, for example, molecules capable of modifying or disrupting transcription and translation of TF-encoding sequences, or molecules which specifically modulate cellular processing or degradation of TF. The present invention also discloses therapeutic compositions and methods for inhibiting or disrupting tumor-host interactions necessary for initiation and maintenance of the metastatic process.</p>		

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INHIBITION OF TUMOR METASTASIS
VIA NEUTRALIZATION OF TISSUE FACTOR FUNCTION

5

TECHNICAL FIELD

The present invention relates to methods and compositions for preventing or treating metastasis. More particularly, the present invention relates to molecules that bind tissue factor (TF) and disrupt the TF·VIIa complex, thereby inhibiting the onset and progression of metastasis. In addition, the present invention relates to molecules capable of disrupting tissue factor expression or function, or the function of TF·VIIa complexes. The present invention also discloses therapeutic compositions and methods for inhibiting or disrupting tumor-host interactions necessary for initiation and maintenance of the metastatic process.

BACKGROUND OF THE INVENTION

Metastasis is a multi-step process which requires highly adapted interactions of tumor cells with host target organs. The metastatic process has specific requirements which must be met by both tumor cells and host factors, in order for the process to be completed successfully.

Hematogenous metastasis leads to disseminated tumor growth in distant sites and is the final stage in the progression of many neoplasias. The metastatic process has been divided into several steps: (1) vascularization of the primary tumor; (2) local invasion and intravasation of tumor cells into the vasculature; (3) survival of tumor cells or tumor cell aggregates in the circulating blood; (4) arrest in the capillary bed of the target organ; (5) extravasation; and (6) proliferation into the target organ (Fidler, I.

J., Cancer Res. 50: 6130-6138 (1990)). Tumor cells can only complete this multi-step process successfully if they have evolved to perform specific functions at each different step and if their specific abilities are
5 complemented by host factors. Clinically and experimentally, different tumors produce metastasis to specific organs, which requires specific contributions from tumor cells and cells in the host organ.

Malignant cells are characterized by their ability
10 to invade locally and their ability to metastasize to distant sites. Tumor cells engage in various highly-adapted interactions with the host to accomplish the multi-step process of metastasis. This involves access of tumor cells to the vasculature by local invasion and
15 entry into the vascular compartment, followed by arrest and implantation of tumor cells in the capillary bed of target organs and subsequent proliferation into metastases (Fidler, Cancer Res. 50: 6130-6138 (1990)).

Melanoma metastasis is a paradigmatic model which
20 is useful in the analysis of the molecular pathways of hematogenous metastasis (Clark, Br. J. Cancer 64: 631-644 (1991)). Clinically, melanoma metastasizes from the skin to a variety of organs, including the lungs (Akslen, et al, Invasion Metastasis 8: 193-204 (1988)).
25 This path of metastasis is recapitulated with many human melanoma cell lines when introduced intravenously into immune deficient mice. (See, eg, Cornil, et al, J. National Cancer Institute 81: 938-944 (1989); Mueller, et al, Cancer Met. Rev. 10: 193-200 (1992).)
30 Recent studies have identified molecular participants in the metastatic process of tumor cells, such as the expression of adhesion receptors (Chan, et al., Science 251: 1600-1602 (1991)), as well as host factors, such as constitutively expressed organ-specific or inducible
35 endothelial cell adhesion molecules (Zhu, et al., PNAS

USA 88: 9568-9572 (1991); Rice, et al., Science 246: 1303-1306 (1989)).

Efficient metastasis of tumor cells has been suggested to be dependent on the plasma coagulation cascades. (See, e.g., Zacharski, et al, Cancer 44: 732-741 (1979); Dvorak, Human Pathol. 18: 275-284 (1987); Murray, Br. J. Cancer 64: 422-424 (1991); Smith, et al, Thromb. Res. 50: 163-170 (1988).) Rather non-selective anticoagulants such as warfarin and heparin can inhibit experimental metastasis of certain tumors in animals (McCulloch, et al, Br. J. Surg. 74: 879-883 (1987); Beuth, et al, Cancer Res. Clin. Oncol. 113: 359-362 (1987)). Studies with the highly-specific thrombin inhibitor hirudin suggest that generation of the pleiotropic serine protease thrombin may trigger essential events during metastasis of melanoma cells (Esumi, et al, Cancer Res. 51: 4549-4556 (1991)).

Other serine proteases of the coagulation system, such as factor VII, have also been suggested as potentially important host factors for metastasis (Francis, et al., Br. J. Cancer 65: 329-334 (1992)). Indeed, factor VII at the site of implantation may bind to tissue factor, its receptor and catalytic cofactor, which can be expressed on host endothelial cells in response to induction by the tumor secreted cytokine vascular permeability factor (Keck, et al., Science 246: 1309-1312 (1989); Clauss, et al., J. Exp. Med. 172: 1535-1545 (1990)). Thus, the formation of an initiating TF·VIIa protease complex on host cells may locally trigger coagulation as an important step for tumor cell implantation and proliferation. However, earlier studies demonstrated expression of TF on a variety of tumor cell lines (Kadish, et al., J. Natl. Cancer Inst. 70: 551-557 (1983); Silberberg, et al., Cancer Res. 49: 5443-5447 (1989); Bauer, et al.,

Thromb. Res. 56: 425-430 (1989)) indicating that host cell TF may not be required at the local site of tumor cell arrest in the vasculature.

TF is a single-chain, 263-amino acid membrane glycoprotein, and its primary sequence indicates structural similarity with the cytokine receptor family (Edgington, et al., Thromb. Haemost. 66: 67-79 (1991)). TF is a transmembrane cell surface receptor, and functions as the receptor and cofactor for factor VIIa (VIIa) to form a proteolytically active TF·VIIa complex on cell surfaces (Ruf, et al., J. Biol. Chem. 267: 6375-6381 (1992); Ruf and Edgington, Thromb. Haemos. 66: 529-533 (1991)). The TF extracellular domain binds factor VIIa and serves as the catalytic cofactor for this serine protease, thereby initiating the plasma coagulation protease cascades (Edgington, et al. (1991), supra). Although alternative pathways exist to activate coagulation, including a tumor cell protease designated cancer procoagulant (Gordon, et al., Thromb. Res. 6: 127-137 (1975)), the in vivo activation of this protease system predominantly proceeds via the TF pathway (Davie, et al., Biochemistry 30: 10363-10370 (1991)).

TF is typically not expressed to any significant degree on cells of the blood or the endothelium, but is constitutively expressed on certain cell types with a differentiation-dependent regulation (Drake, et al., Am. J. Pathol. 134: 1087-1097 (1989)). Promyelocytic leukemias (Bauer, et al., Thromb. Res. 56: 425-430 (1989)) and pancreatic carcinoma cells (Silberberg, et al., Cancer Res. 49: 5443-5447 (1989)) are examples of TF expression by non-differentiated precursors of cell types that normally do not express TF in their fully differentiated state.

Although correlative evidence suggests that

metastatic tumor cells often express TF, it has not previously been established whether the expression of TF is an epiphenomenon or is indeed required for the metastatic phenotype. Analysis of TF expression on melanoma cells is demonstrated herein to follow the progression from normal melanocytes towards melanoma cells with a metastatic phenotype. Furthermore, via use of a xenograft model, it is disclosed herein that the selective inhibition of TF on the surface of melanoma cells abolishes prolonged adherence of these cells in the microvasculature and inhibits pulmonary metastasis, suggesting a functional importance of TF expressed on tumor cells rather than on host cells during the metastatic process.

15 SUMMARY OF THE INVENTION

In contrast to normal melanocytes, malignant metastasizing human melanoma cells express high levels of tissue factor (TF), the major cellular initiator of the plasma coagulation protease cascades. To explore whether TF may contribute to metastatic tumor dissemination, the effect of specific inhibition of TF function on human melanoma metastasis in severe combined immune deficient (scid) mice was analyzed. Using highly characterized antibodies specific for a functional site on TF, it has now been demonstrated that initial adherence is insufficient for successful tumor cell implantation in a target organ. Rapid arrest of human tumor cells in the lungs of mice was not diminished by inhibition of TF. However, inhibition of TF receptor function and consequent reduction in local protease generation abolished prolonged adherence of tumor cells, resulting in significantly reduced numbers of tumor cells retained in the vasculature of the lungs. The growth of pulmonary metastases was also significantly inhibited

by a blocking anti-TF monoclonal antibody and Fab fragments thereof, whereas a non-inhibitory antibody lacked anti-metastatic effects. Cell surface expression of functional TF thus contributes to melanoma progression by allowing metastatic cells to provide requisite signals for prolonged adhesive interactions and/or transmigration of tumor cells across the endothelium resulting in successful metastatic tumor implantation.

A variety of tumor cells have been shown to express tissue factor (TF) and to initiate the coagulation serine protease cascades in vitro and in vivo as evidenced by changes in a variety of markers for a perturbed hemostatic balance. Presently-disclosed correlative evidence further indicates an association of TF with the metastatic phenotype of tumor cells. However, it is also demonstrated herein that TF expression and function represents not merely a correlate of the metastatic phenotype, but a direct causal role in facilitating metastatic implantation and growth of tumors.

It is now shown that a specific inhibitor of TF function, or a specific inhibitor of cell-surface expression of TF, may be used to block metastasis in vivo. Only human melanoma cell lines with the metastatic phenotype were found to also express moderate to high levels of TF. Blocking of tumor cell TF by binding of an inhibitory monoclonal antibody (mAb) prevented metastasis, but the binding of an equally avid and specific non-inhibitory mAb to TF did not have the same effect. Inhibitory monoclonal antibodies disclosed herein recognize functional epitopes on TF, tending to inhibit TF-initiated coagulation of human plasma by more than 90% and inhibiting specific binding of factor VII to cultured

cells expressing TF by more than 80%. The non-inhibitory mAb apparently recognizes a distinct, nonfunctional epitope.

Thus, it seems clear that a function of TF is required for successful implantation of metastatic tumor cells, particularly in view of the presently-disclosed finding that specific interference with TF function, or specific interference with cell-surface expression of TF, profoundly inhibits metastasis.

In addition to monoclonal antibodies to TF, other specific inhibitors of TF function and the TF·VIIa complex also are disclosed to be effective as therapeutic agents useful in the prevention of metastatic spreading of tumors. Targeting tumor cell TF function for therapeutic intervention in the metastatic process is further aided by the lack of TF expression in the vasculature. This would seem to allow efficient and specific intervention without side effects as expected from conventional anticoagulant therapy.

Therefore, in one embodiment, the present invention contemplates a method of preventing or inhibiting metastasis, comprising the administration of a therapeutically effective amount of molecules capable of preventing or inhibiting the expression of human tissue factor (huTF) on the surface of tumor cells. In a preferred embodiment, the molecules are immunoglobulin molecules capable of binding to huTF. In another variation, the invention contemplates a method of preventing or inhibiting metastasis, comprising the administration of a therapeutically effective amount of molecules capable of inhibiting or disrupting huTF function. In one embodiment, the molecules are immunoglobulin molecules. In a preferred embodiment, the immunoglobulin molecules are

antibodies; in other variations, the antibodies are monoclonal or polyclonal.

In various embodiments, monoclonal antibodies may be selected from the group consisting of TF8-5C4, TF8-5G9, TF8-11D12, TF9-1F1, TF9-1D5, TF9-1E7, TF9-1B8, TF9-1B9, TF9-2C4, TF9-2F6, TF9-4D11, TF9-5G4, TF9-5B7, TF9-5C7, TF9-6B4, TF9-6G4, TF9-6C9, TF9-7E10, TF9-8E8, TF9-9E1, TF9-9C3, TF9-9B4, and TF9-10C2. Particularly preferred monoclonal antibodies include TF8-5G9, TF9-6B4, and TF9-6G4. In another variation, methods using monoclonal antibodies capable of immunoreacting with huTF locus I or locus II epitopes, or both, are contemplated.

The invention also contemplates methods utilizing molecules which comprise immunologically active fragments of immunoglobulin molecules. Various embodiments contemplate that the fragments are selected from the group consisting of Fab, Fab', F(ab)₂, and F(v) fragments, and CD-grafted humanized mAbs or fragments thereof.

The present invention also contemplates methods and compositions used to inhibit the cell surface expression of TF. In one embodiment, molecules capable of inhibiting cell surface expression of TF are capable of modulating the expression of nucleotide sequences regulating the expression of TF, particularly huTF. In another variation, the molecules are capable of modulating cellular processing mechanisms or expression levels of TF. Methods of identifying useful, cell-surface expression-inhibiting molecules are also disclosed. In one embodiment, the method may comprise the culturing of tumor cells or other TF-expressing cells; admixing the cells with a test compound -- preferably, one that is non-cytotoxic; incubating the resulting admixture for a predetermined period of time;

and then determining the amount of TF expressed per cell.

Various preferred embodiments contemplate that therapeutically-effective molecules and compositions of the present invention may be administered at various times and dosages, according to the needs of a particular individual receiving treatment. In one variation, administration of therapeutically effective amounts of compounds (e.g., immunoglobulin molecules) according to the present invention begins subsequent to diagnostic procedures indicating the presence of tumors, neoplasias, or other malignancies. In other preferred embodiments, administration of an anti-huTF antibody or antibody-containing composition as described herein occurs within about 48 hours, preferably within about 12-36 hours, more preferably within about 2-8 hours and most preferably immediately prior to or substantially concurrently with invasive surgical procedures directed at diagnosis or removal of a tumor or malignancy.

In one variation, the molecules are administered within about 1-8 hours of invasive surgical procedures directed at diagnosis or removal of a tumor or malignancy. In another embodiment, the molecules are administered substantially concurrently with invasive surgical procedures directed at diagnosis or removal of a tumor or malignancy. In yet another variation, the molecules are administered as a single bolus 30 minutes prior to surgery. In one embodiment, the bolus comprises a dose of about 500 μ g/kg.

The invention further contemplates administration of therapeutically effective amounts of preferred molecules, wherein preferred amounts for administration are in the range of about 0.05 to 20 milligrams per kilogram body weight per day (mg/kg/day). More

preferably, the administered dose is in the range of 0.1 to 10mg/kg/day. In one variation, the molecules are administered intravenously. Other routes/modes of administration are also contemplated. In various
5 preferred embodiments, the therapeutically effective amount is an amount sufficient to produce plasma levels of the effective ingredient (e.g., antibody molecules) of about 0.05 μ g/ml to about 25 μ g/ml; in another embodiment, an effective amount is in the range of
10 about 0.1 μ g/ml to about 10 μ g/ml. In another embodiment, the therapeutically effective amount is an amount sufficient to produce plasma levels of the effective ingredient of about 1 μ g/ml to about 5 μ g/ml.

Therapeutically effective amounts of the
15 presently-disclosed molecules and compositions administrable according to the within-disclosed methods also include amounts sufficient to inhibit, and preferably inhibit by at least about 10-20 percent, more preferably 30 percent, even more preferably by at
20 least about 50 percent, or about 70 percent, and most preferably by at least about 90 percent, the cell surface expression of TF, or its function, on potentially metastatic cells. In another embodiment, a therapeutically effective amount is one sufficient to
25 inhibit binding of huTF to factor VII or VIIa by at least about 40%, more preferably by about 60%, and even more preferably by at least about 80%. The present invention also contemplates the administration of therapeutically effective amounts of the presently-
30 disclosed compounds sufficient to produce similar degrees or percentages of inhibition of a clinically significant increase in the surface expression of TF on potentially metastatic cells, e.g., tumor cells.

In another aspect, the present invention
35 contemplates methods using molecules capable of

inhibiting the binding of factor VII or VIIa to huTF. In one embodiment, the molecules are capable of disrupting huTF·VIIa complex function. In another variation, the molecules are capable of preventing or
5 inhibiting the activation of substrates and the coagulation cascade via binding to TF·VIIa complex.

The present invention also contemplates methods of identifying molecules capable of inhibiting metastasis. In one such method, cells derived from metastatic
10 tissue -- or from another TF-producing source -- are isolated and suspended; the suspension is then incubated with a sample of the molecules for a predetermined period of time, to form an admixture; and the admixture is subsequently assayed for TF specific
15 activity, thereby assessing the ability of the molecules to inhibit metastasis. An assay according to the present invention may comprise (a) incubating the admixture with a TF-specific antibody-containing solution for a predetermined period of time; (b)
20 incubating the second admixture of (a) with a labeled immunoglobulin or a labeled immunoglobulin fragment for a predetermined period of time; and (c) assaying for the presence of labeled TF-antibody complexes. In various embodiments, the source of TF may be tumor
25 cells or other potentially-metastatic tissues, or the source may be normal cells or tissues, which may be processed to achieve various degrees of purity.

In another embodiment, the present invention contemplates a method for identifying compounds or
30 molecules capable of inhibiting, suppressing or preventing the expression of human tissue factor (huTF) on the surface of tumor cells. In one variation, such a method comprises culturing tumor cells; admixing the cells with a compound to be tested -- preferably, a
35 compound which does not affect the viability of the

cells; incubating the admixture for a predetermined period of time; and assaying for or determining the amount of TF expressed per cell.

The present invention also contemplates various therapeutic compositions. In one embodiment, a therapeutic composition comprises, in unit dose form, molecules capable of preventing or inhibiting the expression of huTF on the surface of tumor cells, in a pharmaceutically acceptable excipient. In a preferred embodiment, the molecules are immunoglobulin molecules and are capable of binding to huTF. In another variation, a therapeutic composition of the present invention comprises, in unit dose form, molecules capable of inhibiting or disrupting huTF function, in a pharmaceutically acceptable excipient.

In various aspects of the invention, the molecules may comprise immunoglobulin molecules or immunologically active fragments of immunoglobulin molecules. In alternative embodiments, the molecules comprise anti-huTF antibody molecules, such as polyclonal or monoclonal antibodies, including, for example, monoclonal antibodies selected from the group consisting of TF8-5C4, TF8-5G9, TF8-11D12, TF9-1F1, TF9-1D5, TF9-1E7, TF9-1B8, TF9-1B9, TF9-2C4, TF9-2F6, TF9-4D11, TF9-5G4, TF9-5B7, TF9-5C7, TF9-6B4, TF9-6G4, TF9-6C9, TF9-7E10, TF9-8E8, TF9-9E1, TF9-9C3, TF9-9B4, and TF9-10C2. In another variation, monoclonal antibodies TF8-5G9, TF9-6B4, and TF9-6G4 are particularly preferred. In other variations, the molecules are capable of inhibiting the binding of factor VII or VIIa to huTF, or they are capable of disrupting huTF·VIIa complex function.

Finally, the present invention also contemplates methods of inhibiting huTF synthesis in the vasculature using anti-sense oligonucleotides, as well as methods

and compositions for inhibiting the catalytic site of factor VIIa in the huTF·VIIa complex. Further contemplated is the use of peptidyl and other pharmaceutical compositions to interfere with huTF ↔ VII/VIIa interactions. Disruption or inhibition of the TF pathway using compounds, compositions and methods of the present invention is also contemplated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates TF expression by cultured human melanocytes and melanoma cell lines. The various cell lines used are shown on the vertical axis; procoagulant activity expressed in mU/10⁶ cells is shown on the horizontal axis. TF specific activity per cell line is also illustrated. TF functional activity was analyzed in a plasma coagulation assay after octyl-glucopyranoside lysis of cell pellets of normal human epidermal melanocytes (NHEM) and melanoma cell lines WM35, M24met, and C8161. TF specific activity was assessed after blocking TF in cell lysate by a 30 minute preincubation with mAb TF8-5G9 (30μg/ml). Means from four independent experiments are illustrated.

Figures 2A and B illustrate the surface expression of TF on metastatic melanoma cell lines. In each instance, the number of cells is plotted on the vertical axis, while fluorescence intensity is shown on the horizontal axes. Indirect immunofluorescence and flow cytometry were used to demonstrate TF antigen on the surface of M24met (Fig. 2A) and C8161 (Fig. 2B) melanoma cell lines. Cells were incubated with 50μg/ml isotype matched control Ab (TIB115; solid line), or anti-TF mAb TF8-5G9 (bold line) and TF9-10H10 (dotted line), respectively. Bound mAb was visualized by a secondary goat anti-mouse IgG conjugated to fluorescein isothiocyanate. Results of a representative experiment are shown in each of 2A and 2B.

Figure 3A illustrates functional activity of human TF in human and mouse plasma. Clotting time in seconds is plotted on the vertical axis, whereas the amount of human TF (in ng/ml) is shown on the horizontal axis.

5 The ability of phospholipid reconstituted recombinant human TF to initiate coagulation in mouse plasma (open circles) was evaluated in a standard clotting assay in comparison to pooled normal human plasma (closed circles). Figure 3B shows that, in either case, a
10 preincubation with 30 μ g/ml TF8-5G9 -- but not with TF9-10H10 -- inhibited TF-induced coagulation. In Fig. 3B, procoagulant activity (mU/ml) is shown on the vertical axis. On the horizontal axis, results of preincubation of human vs. mouse plasma with 30 μ g/ml control
15 (TIB115), 10H10 (TF9-10H10) or 5G9 (TF8-5G9) mAb is shown.

Figures 4A and 4B illustrate the distribution of radiolabeled M24met cells after intravenous injection into scid mice. On the vertical axis, time in minutes
20 (4A) or hours (4B) is shown. On the horizontal axis, the percent of injected dose is illustrated, in liver, blood, and lungs. In 4A, $p = 0.018$; in 4B, $p = 0.005$. Cells were labeled with 5-(125 I)-iodo-2'-deoxyuridine for 24 hours, washed and incubated for 45 minutes with
25 1mg monoclonal antibody TF8-5G9 (white bars) or buffer control (dark bars). Radiolabeled M24met cells were injected into the tail vein of scid mice. Mice were bled and sacrificed at different time points, as shown. Means and standard deviations are based on four animals
30 per group at each time interval. The probability of no difference occurring between groups at a given time was assessed with the Wilcoxon rank sum test.

Figure 5 illustrates the ability of anti-TF monoclonal antibodies to inhibit tumor associated TF in
35 vivo and the TF expression of M24met melanoma cells in

vivo. Tissue factor procoagulant activity in mU/mg is indicated on the vertical axis, whereas TF expression in the presence or absence of mAb TF8-5G9 in cultured cells, subcutaneous tumors, and lymph node metastasis is represented by the dark bars (- TF8-5G9) and light bars (+ TF8-5G9) extending vertically from the horizontal axis.

DETAILED DESCRIPTION

A. Definitions

"Amino acid": The amino acid residues described herein are preferred to be in the natural L-configuration. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin binding is retained by the polypeptide. NH₂ refers to the free amino group present at the amino terminus of a polypeptide, while COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, J. Biol. Chem. 243: 3557-59, (1969), abbreviations for amino acid residues are as shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

	<u>SYMBOL</u>		<u>AMINO ACID</u>
	<u>1-Letter</u>	<u>3-Letter</u>	
	Y	Tyr	L-tyrosine
	G	Gly	glycine
	F	Phe	L-phenylalanine
30	M	Met	L-methionine
	A	Ala	L-alanine
	S	Ser	L-serine
	I	Ile	L-isoleucine
	L	Leu	L-leucine
35	T	Thr	L-threonine

	V	Val	L-valine
	P	Pro	L-proline
	K	Lys	L-lysine
	H	His	L-histidine
5	Q	Gln	L-glutamine
	E	Glu	L-glutamic acid
	W	Trp	L-tryptophan
	R	Arg	L-arginine
	D	Asp	L-aspartic acid
10	N	Asn	L-asparagine
	C	Cys	L-cysteine

It should be noted that all amino acid residue sequences are represented herein by formulae whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a bond to a further sequence of one or more amino acid residues up to a total of about fifty residues in the polypeptide chain.

The term "antibody" in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Illustrative antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v).

Fab and F(ab')₂ portions of antibody molecules may be prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well known. (See, e.g.,

U.S. Pat. No. 4,342,566 to Theofilopoulos, et al., the disclosures of which are hereby incorporated by reference.) Fab' antibody molecule portions are also well known and are produced from F(ab')₂ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred, and is utilized as illustrative herein.

The term "antibody combining site" refers to that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) antigen.

The word "antigen" has been used historically to designate an entity that is bound by an antibody, and also to designate the entity that induces the production of the antibody. More current usage limits the meaning of antigen to that entity bound by an antibody, while the word "immunogen" is used for the entity that induces antibody production.

In some instances, the antigen and immunogen are the same entity as where a synthetic polypeptide is utilized to induce production of antibodies that bind to the polypeptide. However, the same polypeptides may also be able to induce antibodies that bind to a whole protein, in which case the polypeptide is both immunogen and antigen, while the whole protein is an antigen. Where an entity discussed herein is both immunogenic and antigenic, it will generally be termed an antigen.

The term "antigenic determinant" refers to the actual structural portion of the antigen that is immunologically bound by an antibody combining site.

The term is also used interchangeably with "epitope".

The term "immunoreact" in its various forms is used herein to refer to binding between an antigenic determinant-containing molecule and a molecule
5 containing an antibody combining site such as a whole antibody molecule or a portion thereof.

As used herein, the term "specifically bound" refers to a non-random binding reaction between a cell surface receptor and a ligand molecule.

10 The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody containing or having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single
15 binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen, e.g., a bispecific (chimeric)
20 monoclonal antibody.

The phrase "monoclonal antibody composition" in its various grammatical forms refers to a population of antibody molecules that contain only one species of antibody combining site capable of immunoreacting with
25 a particular antigen. A monoclonal antibody composition thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody composition may therefore contain an antibody molecule having a plurality of antibody
30 combining sites, each immunospecific for a different antigen, e.g., a bispecific monoclonal antibody.

A monoclonal antibody composition is typically composed of antibodies produced by clones of a single cell called a hybridoma that secretes (produces) but
35 one kind of antibody molecule. The hybridoma cell is

formed by fusing an antibody-producing cell and a myeloma or other self-perpetuating cell line. Such antibodies were first described by Kohler and Milstein, Nature 256: 495-497 (1975), which description is
5 incorporated by reference herein.

A monoclonal antibody composition can also be produced by methods well known to those skilled in the art of producing chimeric antibodies. Those methods include isolating, manipulating, and expressing the
10 nucleic acid that codes for all or part of an immunoglobulin variable region including both the portion of the variable region comprised by the variable region of immunoglobulin light chain and the portion of the variable region comprised by the
15 variable region of immunoglobulin heavy chain. Methods for isolating, manipulating, and expressing the variable region coding nucleic acid in procaryotic and eucaryotic hosts are disclosed in Robinson et al., PCT Publication No. WO 89/0099; Winter et al., European
20 Patent Publication No. 0239400; Reading, U.S. Patent No. 4,714,681; Cabilly et al., European Patent Publication No. 0125023; Sorge et al., Mol. Cell Biol. 4: 1730-1737 (1984); Beher et al., Science 240: 1041-1043 (1988); Skerra et al., Science 240: 1030-1041
25 (1988); and Orlandi et al., PNAS USA 86: 3833-3837 (1989). (The disclosures of the art cited in the present disclosure are hereby incorporated by reference herein.)

Typically, the nucleic acid codes for all or part
30 of an immunoglobulin variable region that binds a preselected antigen (ligand). Sources of such nucleic acids are well known to one skilled in the art and, for example, may be obtained from a hybridoma producing a monoclonal antibody that binds the preselected antigen,
35 or the preselected antigen may be used to screen an

expression library coding for a plurality of immunoglobulin variable regions, thus isolating the nucleic acid.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and their grammatical variations, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal, particularly a human, without the production of allergic or untoward physiological effects such as nausea, dizziness, gastric upset and the like.

The phrase "substantially simultaneously" or "substantially concurrently" is used herein to mean within a time period sufficient to produce concurrent results, e.g., prevention or inhibition of the spread of malignant cells -- i.e., metastasis -- that may occur as a result of an invasive surgical procedure by administration of an anti-huTF antibody, anti-huTF antibody-containing composition, or a subcombination or combination thereof, as described herein.

Also, as used herein, "pg" means picogram, "ng" means nanogram, "μg" means microgram, "mg" means milligram, "kg" means kilogram, "μl" means microliter, "ml" means milliliter, and "l" means liter.

B. Therapeutic Methods and Compositions

1. Methods

The present invention contemplates methods of treating and/or preventing metastasis, and compositions useful therefor. Patients in need of such treatment include those at risk for or suffering from metastatic or potentially-metastatic tumors, malignancies, or neoplasias, such as melanoma and the like. Patients particularly able to benefit from the present invention are those with diagnosed tumors, neoplasias, or other

malignancies, particularly those with a tendency to metastasize.

Thus, in one embodiment, the present invention contemplates a method of preventing or inhibiting metastasis by administering to a patient in need of such treatment a therapeutically effective amount of a molecule that interferes with cell-surface expression of TF or with TF function. Methods of interfering with the cell-surface expression of TF may preferentially utilize molecules or compounds capable of interfering with or modulating the expression of TF-related genes, for example. In general, molecules capable of disrupting cell-surface expression of TF must be of sufficiently small size to facilitate the entry of such molecules into the cell. Also, such molecules are preferably non-toxic to the cells. Molecules capable of disrupting or modulating cell-surface TF expression may include, for example, nucleotide sequences encoding regulatory molecules, or the regulatory molecules themselves. Other examples of molecules which may suppress cell-surface expression of TF include molecules which specifically modulate cellular processing and degradation of TF.

Methods of interfering with TF function may preferentially employ immunoglobulins, such as anti-TF antibodies. A more preferred method utilizes an anti-huTF antibody. An even more preferred method utilizes an inhibitory anti-huTF antibody specific for locus I or II epitopes -- or both -- as described in Ruf, et al., Biochem. J. 278: 729-733 (1991), which is incorporated herein by reference. Essentially, antibodies to the locus I epitope include inhibitory (e.g., TF8-5G9) and non-inhibitory (e.g., TF9-10H10) antibodies. Antibodies to the locus II epitope include inhibitory (e.g. TF9-6B4, TF9-6G4) and non-inhibitory

antibodies as well. The locus I epitope appears to be encoded by exons 4 and 5 of huTF, whereas the locus II epitope appears to be encoded by exon 3 of huTF.

Applicants do not intend, however, for the present invention to be limited by a particular hypothesis or theory of operation.

Exemplary antibody molecules include intact immunoglobulin molecules, substantially intact immunoglobulin molecules, and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F(ab)₂ and F(v).

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to inhibit, and preferably inhibit by at least about 10 percent, more preferably by about 20-30 percent, even more preferably by at least about 50 percent, or about 70 percent, and most preferably by at least 90 percent, a clinically significant increase in the surface expression of TF on potentially metastatic cells, e.g., tumor cells. Preferred therapeutically effective amounts for the agents used herein as active ingredients are provided below. A clinically significant increase in the cell surface expression of TF on tumor cells is an increase of about 10 times the normal level to about 50,000 times the normal level. Often, a clinically significant increase is an increase of about 100 to 1,000 times the normal level of surface-expressed huTF. Methods for determining the level of TF on cell surfaces are well known in the art; particularly preferred methods are those described herein, in Section H. A therapeutically effective amount of the agents used herein may also comprise an amount sufficient to inhibit about 1-20 percent, and preferably inhibit by about 20-50 percent, more

preferably by about 40-70 percent, even more preferably by about 80-100 percent, the functional activity of huTF. Methods of assessing the functional activity of TF are known in the art; a preferred method is that
5 described hereinbelow in Section G.

In another embodiment, the method comprises administering to a patient in need of treatment (i.e., with metastatic tumors or at risk for metastasis) a therapeutically effective amount of an anti-human TF
10 (huTF) antibody, preferably an amount sufficient to inhibit huTF functional activity in vivo. In yet another variation, the method comprises administering to a patient in need of treatment a therapeutically effective amount of a molecule capable of inhibiting or
15 preventing the cell-surface expression of huTF.

In another embodiment, a therapeutic method of the present invention comprises administering a therapeutically effective amount of an anti-human TF antibody, preferably an affinity-purified polyclonal
20 antibody, and more preferably a mAb. In addition, the anti-huTF antibody molecules used herein may be in the form of Fab, Fab', F(ab')₂ or F(v) portions of whole antibody molecules. Preferably, the amount of anti-huTF antibody administered is sufficient to reduce by
25 at least about 20 percent, preferably by at least 40 percent, and more preferably by at least 60 or 80 percent, a clinically significant increase in the levels of tumor cell surface-expressed, functional huTF in a patient diagnosed as having tumors, malignancies,
30 or neoplasia, or displaying at least one of the symptoms associated therewith. As previously discussed, patients capable of benefiting from this method include those suffering from malignancies in which metastasis is not uncommon.

35 Methods for isolating TF and for inducing anti-

huTF antibodies are well known in the art. (See, for example, U.S. Patent No. 5,110,730 to Edgington, et al.) Various methods for determining and optimizing antibody affinities and inhibitory abilities are also known in the art. Preferred methods of determining and optimizing the ability of an anti-huTF antibody to inhibit the binding of huTF or the formation of TF·VIIa complexes on cell surfaces and thereby inhibit TF-induced metastasis are discussed in Section G and in the Examples hereinbelow.

2. Compositions

Useful compositions disclosed herein include, as an active ingredient, molecules capable of inhibiting or preventing the cell-surface expression of huTF. Molecules capable of disrupting or modulating cell-surface TF expression may include, for example, nucleotide sequences encoding regulatory molecules, or the regulatory molecules themselves. Other examples of molecules which may suppress cell-surface expression of TF include molecules which specifically modulate cellular processing and degradation of TF.

Compositions of the present invention also include those in which the active ingredient is capable of inhibiting TF function. Preferred active ingredients according to the present invention include, for example, immunoglobulins and fragments thereof, including antibodies and antibody fragments.

Preferably, an anti-huTF antibody used in a therapeutic method of this invention is an affinity purified polyclonal antibody. More preferably, the antibody is a monoclonal antibody (mAb). In addition, preferred anti-huTF antibody molecules used herein may be in the form of Fab, Fab', F(ab')₂ or F(v) portions of whole antibody molecules.

Preferred monoclonal antibodies useful in

practicing the present invention are those capable of being produced by a hybridoma such as hybridoma TF8-5G9 or TF9-6B4, described in U.S. Pat. No. 5,110,730 to Edgington, et al. While mAbs such as TF8-5G9 and TF9-6B4 can be produced by hybridoma culture, the invention is not so limited. Also contemplated is the use of mAbs produced by an anti-TF immunoglobulin expressing nucleic acid cloned from a hybridoma such as TF8-5G9, TF9-6B4, or TF9-6G4. That is, the nucleic acid expressing the anti-TF antibody molecules secreted by hybridomas TF8-5G9, TF9-6B4, or TF9-6G4 and the like can be transferred into another cell line to produce a transformant. The transformant is genotypically distinct from the original hybridoma but is also capable of producing anti-huTF antibody molecules, including immunologically active fragments of whole antibody molecules, corresponding to those secreted by the hybridoma. See, for example, United States Patent No. 4,642,334 to Reading; PCT Publication No. WO 890099 to Robinson et al.; European Patent Publications No. 0239400 to Winter et al. and No. 0125023 to Cabilly et al.

Preferred antibodies or fragments thereof display an immunoreactivity for huTF that is similar to that of those produced by the above-described hybridomas. As used herein, the term "immunoreactivity" in its various grammatical forms refers to the concentration of antigen required to achieve a 50% inhibition of the immunoreaction between a given amount of the antibody and a given amount of huTF antigen. That is, immunoreactivity is the concentration of antigen required to achieve a B/B_0 value of 0.5, where B_0 is the maximum amount of antibody bound in the absence of competing antigen and B is the amount of antibody bound in the presence of competing antigen, and both B_0 and B

have been adjusted for background. (See, e.g., Robard, Clin. Chem. 20: 1255-1270 (1974).)

Preferred anti-huTF antibodies according to the present invention also include antibodies that inhibit or interfere with huTF function. Such preferred antibodies include antibodies to huTF which are capable of inhibiting the binding of factor VII or VIIa. More preferred are huTF antibodies which are specific for primates, and more preferably humans (i.e., such antibodies do not cross-react with TF from non-primates, and more preferably, do not cross-react with TF from non-human primates). Exemplary preferred monoclonal antibodies are listed in Table 1 below.

Table 1
Monoclonal Antibodies to huTF*

	<u>Name</u>	<u>Isotype</u>	<u>% Inhibition of:</u>		<u>Inhib. of</u>
			<u>Coag.^a</u>	<u>VII^b</u>	<u>primate TF</u>
5	TF8-5C4	IgG ₁ , kappa	96	57	--
	TF8-5G9	IgG ₁ , kappa	99	80	--
	TF8-11D12	IgG ₁ , kappa	99	82	--
	TF9-1F1	IgG ₁ , kappa	95	83	M, B ^c
	TF9-1D5	IgG ₁ , kappa	95	76	M, B
10	TF9-1E7	IgG ₁ , kappa	97	90	M, B
	TF9-1B8	IgG ₁ , kappa	98	83	M, B
	TF9-1B9	IgG ₁ , kappa	97	84	M, B
	TF9-2C4	IgG ₁ , kappa	97	78	M, B
	TF9-2F6	IgG ₁ , kappa	97	79	M, B
15	TF9-4D11	IgG ₁ , kappa	97	81	M, B
	TF9-5G4	IgG ₁ , kappa	97	83	M, B
	TF9-5B7	IgG ₁ , kappa	97	74	M, B
	TF9-5C7	IgG ₁ , kappa	96	72	M, B
	TF9-6B4	IgG ₁ , kappa	96	98	M, B
20	TF9-6G4	IgG ₁ , kappa	95	78	M, B
	TF9-6C9	IgG ₁ , kappa	95	47	--
	TF9-7E10	IgG ₁ , kappa	97	54	--
	TF9-8E8	IgG ₁ , kappa	97	76	M, B
	TF9-9E1	IgG ₁ , kappa	90	71	M, B
25	TF9-9C3	IgG ₁ , kappa	97	82	M, B
	TF9-9B4	IgG ₁ , kappa	95	82	M, B
	TF9-10C2	IgG ₁ , kappa	98	71	M, B
	TF9-10H10	IgG ₁ , kappa	0	20	--

- 30 ^a Inhibition of coagulation of human plasma induced by purified human brain TF.
- ^b Inhibition of specific ¹²⁵I-factor VII binding to J82 cells.
- ^c Inhibition of coagulation of human plasma induced by B (crude baboon brain extract) or M (lysed COS cells -- African green monkey
- 35

kidney cells). A letter is entered for a species if the antibody inhibited the procoagulant activity by $\geq 60\%$.

* (Adapted from Morrissey, et al., Thromb. Res. 52: 247-261 (1988).)

5

Other preferred anti-huTF antibodies useful in practicing the present invention may also immunologically cross-react with a peptide analog of TF, and more preferably, an analog of huTF. A "TF peptide analog" is a polypeptide capable of competitively inhibiting the binding of factor VII or VIIa to TF expressed on the surface of tumor cells. Preferred TF peptide analogs include the epitopes identified as locus I or locus II, or both. Other preferred anti-huTF antibodies useful in practicing the present invention immunologically cross-react with recombinant TF or with TF mutants which are functionally similar to, or include the same functional epitope as, native huTF. Such mutant TFs include, without limitation, TF_{A165A166} (Ruf, et al., J. Biol. Chem. 267: 6375-6381 (1992)).

Monoclonal antibody compositions are also contemplated by the present invention. A monoclonal antibody composition contains, within detectable limits, only one species of antibody combining site capable of effectively binding huTF. Thus, a monoclonal antibody composition of the present invention typically displays a single binding affinity for huTF, even though it may contain antibodies capable of binding proteins other than huTF. In one embodiment, a monoclonal antibody composition contains antibodies capable of inhibiting the function of huTF. In another embodiment, a monoclonal antibody

composition contains antibody molecules that immunoreact with huTF and a polypeptide analog of the tissue factor epitope located near the carboxy terminus of huTF, preferably p106-219, and more preferably, the locus I epitope. In another preferred embodiment, the antibody molecules immunoreact with huTF and a polypeptide analog of the tissue factor epitope identified as the locus II epitope. The antibody molecules contained in these compositions immunoreact with huTF at or near its locus I or II epitopes and thereby neutralize its functionality and/or its ability to bind factor VII or VIIa.

A monoclonal antibody composition of the present invention can be produced by initiating a monoclonal hybridoma culture comprising a nutrient medium containing a hybridoma that secretes antibody molecules of the appropriate polypeptide specificity. The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibody molecules into the medium. The antibody-containing medium is then collected. The antibody molecules can then be further isolated by well-known techniques.

Many techniques are also available for generating antibodies to specific cell surface receptors; such antibodies may be useful when administered in conjunction with, or conjugated to, anti-TF antibodies of the present invention. Examples of known methods of generating antibodies to cell surface receptors include U.S. Patent No. 4,816,567 and published PCT application no. WO90/14424. Various methods of targeting specific cells using antibodies are also known; see, e.g., Huang, et al., J. Biol. Chem. 255: 8015-81 (1980). (References cited herein are incorporated by reference.)

Media useful in the preparation of these compositions are both well-known in the art and commercially available, and include synthetic culture media, inbred strains of mice, and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco, et al., Virol. 8: 396 (1959)) supplemented with 4.5g/l glucose, 20mM glutamine, and 20% fetal calf serum. An exemplary inbred mouse strain is the BALB/c strain. The monoclonal antibody compositions produced by the above methods can be used, for example, in diagnostic and therapeutic modalities wherein formation of an huTF-containing immunoreaction product is desired.

The preparation of therapeutic compositions which contain polypeptides, antibody molecules, or portions thereof as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, or pH buffering agents which enhance the effectiveness of the active ingredient.

A therapeutic composition of the present invention may also take the form of tablets, pills, capsules, sustained release formulations, or powders. In any case, the compositions preferably contain from about

10%-95% active ingredient, and more preferably, about 25%-70% active ingredient.

A polypeptide or antibody can be formulated into the therapeutic composition as neutralized
5 pharmaceutically acceptable salt forms.

Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example,
10 hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric
15 hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The therapeutic polypeptide- or antibody-containing compositions are conventionally administered
20 intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined
25 quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a
30 therapeutically effective amount. The quantity of the effective ingredient to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of inhibition or neutralization of TF-binding capacity
35 desired. Precise amounts of active ingredient required

to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of one to several milligrams of active ingredient per individual per day, preferably one to several milligrams of active ingredient per kilogram bodyweight of individual per day, more preferably about 0.05mg/kg/day to about 20mg/kg/day, and even more preferably about 1mg/kg/day to about 5mg/kg/day, and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. For example, a single bolus of about 500µg/kg may be administered (e.g., intravenously) for therapeutic benefit.

Alternatively, continuous modes of administration, such as via intravenous infusion, sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated. In one embodiment, a therapeutically effective amount for intravenous administration is an amount sufficient to produce plasma levels of the effective ingredient (e.g., compounds which bind to TF, such as monoclonal antibodies) of about 0.1µg/ml to about 10µg/ml are contemplated. In another embodiment, administration of an amount sufficient to produce plasma levels of said molecules of about 1µg/ml to about 5µg/ml are contemplated.

3. Conjugates

Within the body, the primary mode of communication and delivery of substances is via the circulatory system. In general, the circulatory system comprises the blood vascular system and the lymphatic system. Of

particular interest are the capillaries.

Capillaries are typically comprised of simple endothelial cells that connect the arterial and venous sides of the circulatory system. Meshes of the capillary network are present throughout the body, varying in size and in shape in different tissues and organs. The intensity of metabolism in a region generally determines the closeness of the mesh. Therefore, there is a close network in the lungs, liver, kidneys, mucous membranes, glands, and skeletal muscle, as well as in the grey matter of the brain. The network has a large mesh and is sparse in tissues such as tendons, nerves, smooth muscle, and serous membranes.

The ability to transfer substances through the wall of capillaries is referred to as permeability. Permeability varies regionally and, under changed conditions, locally.

In general, it is agreed that tumors must induce a new blood supply if they are to grow beyond a diameter of a few millimeters, and a great deal of attention has been focused on the mechanisms by which tumors induce angiogenesis. (For example, see Folkman, Adv. Cancer Res. 43: 175-203 (1985).) Significant attention has also been devoted to the anatomy and physiology of the new blood vessels that come to supply tumors. (Id.)

It is generally agreed that tumor vessels are anatomically heterogeneous structures. Often, they consist of relatively undifferentiated channels, lined by a simple endothelium and with fewer pericytes and smooth muscle cells than would be expected of comparably sized vessels in normal tissues. One property of tumor vessels on which most investigators agree is that, relative to normal vessels, tumor vessels are hyperpermeable to circulating

macromolecules. This observation demands explanation because of its obvious relevance to an understanding of the localization of monoclonal antibodies and tumoricidal drugs in solid tumors. (See, e.g., Dvorak, et al., Am. J. Pathol. 133: 95-109 (1988).) Whereas small molecules pass freely through normal capillaries and other vessels with intact interendothelial cell junctions, the permeability of the normal vasculature to macromolecules is tightly regulated. Normally, macromolecules are largely retained within the circulation and the small amounts that do escape are thought to do so by means of vesicular transport or by the formation of transient transcytoplasmic channels across endothelial cells. (See, e.g., Milici, et al., J. Cell Biol. 105: 2603-2612 (1987).) In inflammation, however, the escape of macromolecules is greatly increased; agonists such as histamine provoke a contraction of post-capillary endothelial cells, resulting in the formation of interendothelial cell gaps through which macromolecules and even particulates may escape. This suggests, therefore, that tumor vasculature is "leaky".

Studies show that uptake of antibodies by tumor tissue correlates well with vascular permeability and blood flow (Sands, et al., Cancer Res. 48: 188-193, (1988)). A similar study indicates that administration of a vasoactive agent may under some circumstances increase the perfusion of tumor relative to other tissues and increase tumor uptake and concentration of pharmaceuticals. (Bomber, et al., J. Nucl. Med. 27: 243-245 (1986)).

Thus, it may also be appropriate to enhance delivery of the immunoglobulins of the present invention by administering them in conjunction with, or by conjugating them to, other therapeutically useful

agents. For example, the anti-huTF mAbs of the present invention may be administered in conjunction with one or more vasoactive agents.

Preferably, immunoglobulin-agent conjugates would
5 retain the ability to bind with antigen; more preferably, they would also fail to bind with any component of blood or normal, intact, non-inflamed endothelium. It is also preferable that such
10 conjugates would show little or no tendency to pass across the endothelium of normal blood vessels from blood into tissues. It is also conceivable that anti-huTF mAbs of the present invention may be conjugated to other antibodies or fragments thereof, such as mAbs
15 that are specifically targeted to an antigen present on a specific type of tumor. Methods for making and using such conjugates are known in the art and will thus not be discussed at length herein.

Vasoactive agents which improve the extravascular penetration and binding of monoclonal antibodies (as
20 well as other drugs or molecules) may also be useful and may be administered in conjunction with, or conjugated to, the immunoglobulins of the present invention. Although conjugates other than vasoactive agent-immunoglobulin conjugates are also contemplated
25 by the present invention, immunoglobulin-vasoactive agent conjugates (or "immunoconjugates") shall be discussed herein as exemplary. It is expressly to be understood that the present invention is not limited to such conjugates.

30 Immunoconjugates useful as disclosed herein may be prepared by various methods including genetic approaches, or by covalently or otherwise linking a selected clinically useful immunoglobulin to a selected biologically active agent which is inflammation
35 provoking, and preferably vasoactive. The linking

agent and the chemical procedure of assembling the immunoconjugate should be selected and carried out so as not to compromise the effectiveness of the antibody in binding to its target or the effectiveness of the vasoactive agent in stimulating natural defense mechanisms.

Vasoactive agents as described herein are typically used to increase the flow of blood and/or the vessel permeability in the tumor so as to improve the extravascular penetration and binding of monoclonal antibodies and other drugs or molecules in vivo. They may act directly by increasing the volume of tumor blood flow or the degree of tumor blood vessel "leakiness," or indirectly by inducing an inflammatory immune response at the tumor site.

Inflammation can be induced by chemotactic factors which attract polymorphonuclear leukocytes, macrophages, eosinophils, basophils, mast cells, T-cells and other cells associated with inflammation. These cells, when stimulated, secrete immunomodulatory factors which then act on the tumor blood flow and blood vessel permeability to increase the percent of the injected dose penetrating and binding to the tumor.

Vasoactive agents having the described reactivity at the tumor site and suitable for linking to monoclonal antibodies in an immunoconjugate are found in several biochemical classes, including peptides, carbohydrates, and lipids, and their derivatives. Examples of useful agents include tachykinins, leukotrienes, anaphylatoxins, lymphokines, inflammagens, human eosinophil acidic tetrapeptides, proteases, platelet-activating factor (PAF), prostaglandins, prostaglandin derivatives, histamine, and vasoactive carbohydrate compounds such as glucan. In all instances, vasoactive agents are selected

according to availability and applicability to the stated goals of therapy or study.

The structural link between the immunoglobulin and the agent to which it is conjugated, and the chemical method by which they are joined, should be chosen so that the binding ability of the immunoglobulin and the biological activity of the agent, when joined in the conjugate, are minimally compromised. Some of the available conjugation methods known in the art include the following.

ECDI (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) produces crosslinks between the antibody and a second molecule (or conjugant), regardless of either molecule's orientation. Conjugates are derived by condensation of the antibody and conjugant under acidic conditions with ECDI. This method provides a rapid and simple means of conjugation. (See Goodfriend, et al., Science 144: 1344-1346 (1964).)

N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) is a heterobifunctional reagent which introduces thiol groups to the terminal amino of proteins, and has been used in a number of immunoconjugates. (Carlsson, et al., Biochem. J. 173: 723-737 (1978).) The cis-aconitic linkage described by Shen, et al has the characteristic of releasing a conjugant at low pH, such as in a secondary lysosome following endocytosis of the receptor-bound antibody molecule. The method allows conjugation to the carbohydrate side groups of the antibody molecules. (Shen, W.-C., and Ryser, Biochem. Biophys. Res. Comm. 102(3): 1048-1054 (1981).)

Periodate oxidation can be used to oxidize and cleave carbon-carbon bonds in a sugar ring. The exposed terminal groups can then link to NH_2 groups on proteins in a Schiff base linkage which is reduced with NaBH_4 . (Kitao and Hattori, Nature 265, January 6, pp.

81-82 (1977).) N-hydroxysuccinimide (NHS) activates a terminal COOH group, for example, of a peptide, to form an active ester derivative that can be covalently coupled to the protein of the monoclonal antibody.

5 This method has been used to attach 30 molecules of chlorambucil/antibody with little loss of binding activity. (Smyth, et al., J. Natl. Cancer Inst. 76(3): 503-510 (1986).)

As an alternative method to the chemical linkage
10 of biologically active agents (e.g., vasoactive agents) to immunoglobulins of the present invention, the genetic sequence of the agent can be engineered into the genetic sequence of the immunoglobulin. For example, the genetic sequence of a vasoactive peptide
15 may be engineered into that of one of the antibodies of the present invention.

The dose of vasoactive agent or immunoconjugate to be given is based on criteria of medical judgment and experience, both objective and subjective. However, an
20 adequate measure of an effective dose is that amount required to increase localization of a subsequently- or concurrently-administered diagnostic or therapeutic agent (such as the immunoglobulins of the present invention) to an extent which improves the clinical
25 efficacy of therapy, or accuracy of diagnosis, to a statistically significant degree. Comparison is typically made between treated and untreated tumor host animals to whom equivalent doses of the diagnostic or therapeutic agent are administered.

30 The immunodiagnostic dose may comprise an immunoglobulin having a specificity for huTF and having a label which is detectable in vivo. In a preferred embodiment, this label comprises a radioactive isotope. The immunotherapeutic dose may similarly comprise a
35 clinically useful immunoglobulin such as those

useful as disclosed herein. Antibody TF9-10H10 is secreted by hybridoma TF9-10H10, which was deposited with the ATCC on March 27, 1987, pursuant to Budapest Treaty requirements, and was assigned accession number
5 HB9383.

D. Xenograft Model

Metastasis is a multi-step process that has specific requirements which must be met by both tumor cells and host factors, in order for the process to be
10 completed successfully. Some studies have attempted to correlate the proteases of the coagulation cascade with hematogenous metastasis, but until the present disclosure, it was not clear how and where this proteolytic activity is generated in the metastatic
15 process.

The study of a xenograft model for hematogenous melanoma metastasis allows us to distinguish the contribution of tumor cells to the activation of coagulation from that of host cells, because the
20 antibodies that were used to block procoagulation activity are highly specific for human TF (Ruf, et al., Thromb. Haemost. 66: 529-533 (1991)). Evaluating a small panel of human cell lines, it has now been discovered that the expression of TF increases in
25 melanoma progression and, in particular, that cell lines derived from melanoma metastases have potent procoagulant activity that is entirely TF-dependent. Procoagulant activity not only seems to be required for M24met metastasis but for hematogenous metastasis of
30 other melanoma cell lines. The presently-described experiments using the xenograft model demonstrate that TF function is required for melanoma cells to initiate or undergo hematogenous metastasis.

It is now reported herein that inhibition of TF
35 activity on the surface of metastatic melanoma cell

lines (e.g., M24met and C8161) with specific monoclonal antibodies inhibits experimental pulmonary metastasis in xenograft models. The number of tumor foci in the lungs is significantly reduced ($p < 0.002$) after exposing
5 tumor cells to the monoclonal antibody TF8-5G9 or its Fab fragment, which bind to human TF and inhibit TF function. There was, however, no effect on metastasis when cells were exposed to the monoclonal antibody TF9-10H10, which is directed to a partly-overlapping
10 epitope on TF, but which does not inhibit TF function. Monoclonal antibody TF9-10H10 is further described and characterized in U.S. Patent No. 5,110,730 to Edgington, et al. (which has been incorporated herein by reference).

15 Distribution and fate of radiolabeled tumor cells after I.V. injection indicated at least two phases of melanoma cell arrest in the microvasculature of the lungs. Thus, initial adherence is not influenced by specific inhibitors of tumor cell TF and is apparently
20 not sufficient for prolonged tumor cell survival. Inhibition of TF -- and thus, inhibition of the local generation of proteolytic activity -- abolishes adherence of melanoma cells at least up to four hours after injection. The within-disclosed data also
25 suggest that early and local generation of proteases -- e.g., factor IXa, factor Xa, thrombin -- is required for prolonged adhesive interaction and/or trans-migration of melanoma cells across the endothelium leading to the growth of metastatic foci.

30 E. Cell Lines and Tissue Culture

Normal human epidermal melanocytes (NHEM) were purchased from Clonetics (San Diego, CA). They were maintained in melanocyte growth medium (MGM - Clonetics, San Diego, CA) and used prior to the tenth
35 passage. The human melanoma cell line WM35 has been

established from an early primary melanoma (Kath, et al., Cancer Therapy Control 1: 179-191 (1990)). WM35 was maintained in MCDB153/L15 medium supplemented with insulin (5 µg/ml) and two percent fetal bovine serum (FBS). The human melanoma cell line M24met has been described in detail (Mueller, et al., Cancer Res. 51: 2193-2198 (1991)). The M24met line was derived from M24, which was established from a lymph node metastasis (Guiliano, et al., Cancer Immunol. Immunother. 10: 243-249 (1981); Tsuchida, et al., J. Natl. Cancer Inst. 78: 55-59 (1987)). The human melanoma cell line C8161 was also derived from a metastatic melanoma (Welch, et al., Int. J. Cancer 47: 227-237 (1991)). M24met and C8161 were maintained in RPMI 1640 tissue culture medium supplemented with 10% FBS (Sigma, St. Louis, MO). WM35 and M24met were cultured on tissue culture plastic that was pretreated with 1% gelatin to enhance cellular attachment. All cells were routinely tested from mycoplasma contamination.

20 F. Monoclonal Antibodies

The murine anti-human TF monoclonal antibodies TF8-5G9 and TF9-10H10 have been previously characterized (Ruf, et al., Thromb. Haemost. 66: 529-533 (1991); Ruf, et al., Biochem. J. 278: 729-733 (1991)). Both mAbs are of IgG1 isotype. The IgG1 mAb to the SV40 large T viral antigen (TIB115, ATCC, Rockville, MD) was used as isotype matched irrelevant control. The mAbs were purified on protein A, and Fab fragments of mAb TF8-5G9 were produced by papain digestion (Ruf, et al., Thromb. Haemost. 66: 529-533 (1991)), followed by ion exchange chromatography to remove uncleaved IgG and F_c fragments.

30 G. Functional Analysis of TF

TF functional activity was analyzed in a plasma coagulation assay after octyl-glucopyranoside lysis of

cell pellets, as previously described (Ruf, et al., J. Biol. Chem. 267: 6375-6381 (1992)). Briefly, cells were lysed at 2×10^6 cells/ml with 15mM octyl- β -D-glucopyranoside in HEPES-buffered saline (HBS) at 37°C for 20 minutes, followed by a 3-fold dilution with HBS (see also Drake, et al., J. Cell. Biol. 109: 389-395 (1989)). Clotting times were determined for the cell lysates in a one-stage clotting assay (equal volumes of sample, plasma, and 20mM CaCl_2) and converted to units based on a calibration curve using purified and phospholipid-reconstituted TF. The clotting times obtained in the one-stage clotting assay were converted into units of TF activity using a calibration curve based on serial dilutions of a cell line expressing wild-type TF, as previously described in detail (Rehemtulla, et al., J. Biol. Chem. 266: 10294-10299 (1991)).

TF was quantified by converting clotting times into procoagulant activity units based on a calibration curve using purified and phospholipid reconstituted recombinant TF. The activity which yields a 50s clotting time was defined as the unit 1U/ml. The procoagulant activity was demonstrated to be TF-specific by blocking TF function in the cell lysates by a 30-minute preincubation with mAb TF8-5G9 (30 μ g/ml). The functional activity of human recombinant TF was compared in mouse plasma (Sigma, St. Louis, MO) and pooled normal human plasma using a standard coagulation assay.

30 H. Analysis of Cell Surface TF

The cell surface expression of TF was determined via flow cytometry, as described herein. Melanoma cells (10^6 cells/tube) were suspended in phosphate buffered saline (PBS) with 50 μ g/ml mAb for 45 minutes on ice. Cells were washed, and bound primary mAb was

detected by incubation with goat anti-mouse IgG antibody labeled with fluorescein for 45 minutes on ice. After washing to remove unbound secondary antibody, cells were analyzed with a FACS 440 flow cytometer. Cell surface TF was quantified on M24met melanoma cells by radioligand analysis. The mAb TF8-5G9 was labeled to a specific activity of 5 nCi (185 Bq)/ng with Na¹²⁵I (Amersham, Arlington Heights, IL) with Enzymobeads (BioRad, Richmond, CA). Radioligand binding analysis with the radiolabeled mAb was performed on melanoma cell monolayers as described (Mueller, et al., J. Immunol. 144: 1382-1386 (1990)). The number of TF molecules per cell and the dissociation constant of the ligand were determined by Scatchard plot analysis.

I. TF Expression

Another aspect of the present invention discloses methods of identifying compounds which may be useful as inhibitors of cell-surface expression of TF. In general, an assay for identifying such compounds may comprise the steps of: (a) culturing tumor cells; (b) admixing said tumor cells with a test compound, preferably one that is not cytotoxic; (c) incubating the resulting admixture for a predetermined period of time; and (d) determining the amount of TF expressed (e.g., per cell).

Cell surface expression may be impacted at several levels; depending upon the manner in which one intends to disrupt cell surface expression, a test compound will have different characteristics. For example, if one intends to alter the rate of transcription or translation of a TF gene and/or sequences involved in a TF-regulatory pathway, a "test" compound may comprise a nucleotide sequence such as an antisense nucleotide sequence. Alternatively, it may comprise a molecule

which binds to at least a portion of the TF-encoding sequence or a nucleotide sequence which affects the rate or frequency of transcription of a TF-encoding sequence, such as the TF promoter or regulatory enhancer elements. Such molecules would thus include transcription repressors. Other potentially-useful molecules include molecules that mimic growth factors or otherwise compete with same for binding sites on corresponding cognate cellular receptors, to inhibit the initiation of transcription of the TF gene. (See, e.g., Edgington, et al., Thromb. Haem. 66: 67-79 (1991); Lau, et al., PNAS USA 84: 1182-6 (1987).) Molecules which alter the post-translational regulation of TF expression are also potentially useful as therapeutic agents.

Should one desire to modulate TF cellular processing mechanisms, potentially-useful molecules would likely include those that impact sequestration, for example. Other potentially-useful candidates include molecules which alter TF degradation mechanisms.

Additional molecules or compounds potentially useful as modifiers of expression levels of TF include such substances as interleukins (e.g., IL-1) and other cytokines, as well as growth factors and substances such as tumor necrosis factor (TNF), which exhibit cytokine-like effects. For example, in Bevilacqua, et al., J. Exp. Med. 160: 618-623 (1984), the induction of biosynthesis and cell surface expression of procoagulants via use of IL-1 was described. (Also see Edgington, et al., Thromb. Haem. 66: 67-79 (1991).) Other selected agonists such as endotoxins (e.g. LPS), antigen-antibody complexes, and vascular permeability factor (VPF) may induce expression of TF and may thus be useful as disclosed herein. (See Morrissey, et al.,

in Oxford Surveys on Eukaryotic Genes, MacLean (ed.), Oxford Univ. Press, 1989, p. 67-84; Levy, et al., J. Exp. Med. 154: 1150-1163 (1981); Keck, et al., Science 246: 1309-1312 (1989).)

5

EXAMPLES

The following examples are intended to illustrate, but not limit, the present invention.

Example I

Experimental Metastasis Assay

10 Human melanoma cells (0.5 to 1.0×10^6 cells in $200\mu\text{l}$ PBS) were injected into the lateral tail vein of 6-8 week old female C.B-17 scid/scid mice (from The Scripps Research Institute Rodent Breeding Facility, La Jolla, CA). Prior to injection, cells were incubated
15 for 45 minutes at room temperature. Buffer controls were incubated in PBS and a total of 1mg mAb or Fab fragment was present in the experimental groups. When a 2mg total dose was administered, mice received a second intravenous injection of 1mg mAb after 24 hours.
20 Twenty-one days later, these animals were sacrificed and necropsy was performed. All major organs were systematically examined for the presence of tumors. The lungs were removed and their weight determined prior to fixation in Bouin's solution. Tumor foci on
25 the lung surface were counted under a low magnification microscope. Data were analyzed for statistical significance by the non-parametric Wilcoxon rank sum test.

Example II

Determination of Distribution of Radiolabeled Melanoma Cells After Intravenous Injection

30 The initial localization of radiolabeled tumor cells to organs was analyzed using the methodology described by Esumi, et al. (Cancer Res. 51: 4549-4556
35 (1991)). Cells were cultured for 24 hours in the

presence of 0.1 μCi (3.7 kBq)/ml 5-(^{123}I)-iodo-2'-deoxyuridine (Amersham, Arlington Heights, IL) resulting in the incorporation of 50,000 cpm per 10^6 M24met cells. This had no effect on the viability of the cells. After incubation with mAb or saline for 45 minutes, 10^6 cells in 200 μl were injected into the lateral tail vein of female scid mice. Mice were bled from the orbital sinuses (50 μl /mouse) and sacrificed at 10 min., 90 min., 240 min. and 24 hours post-injection. Lungs, livers, spleens, and kidneys were collected and washed in 70% ethanol for 3 days prior to gamma-counting.

Example III

Assay for Expression of TF in Metastatic Melanomas

The expression of TF on cultured melanocytes and melanoma cell lines was analyzed with a sensitive coagulation assay (see Fig. 1). The various cell lines used are shown on the vertical axis; procoagulant activity expressed in $\text{mU}/10^6$ cells is shown on the horizontal axis. TF functional activity was analyzed in a plasma coagulation assay after octyl-glucopyranoside lysis of cell pellets of normal human epidermal melanocytes (NHEM) and melanoma cell lines WM35, M24met, and C8161. TF specific activity was assessed after blocking TF in cell lysate by a 30 minute preincubation with mAb TF8-5G9 (30 $\mu\text{g}/\text{ml}$). Means from four independent experiments are illustrated in Figure 1.

The procoagulant activity of the cell lysates was shown to be TF-specific by inhibition with anti-TF monoclonal antibodies (see Fig. 1). Both cultured human melanocytes and the cell line WM35, which was derived from a primary non-metastatic melanoma (Kath, et al., Cancer Therapy Control 1: 179-191 (1990)), expressed TF at very low levels. In contrast, the

melanoma lines M24met (Mueller, et al., Cancer Res. 51: 2193-2198 (1991)) and C8161 (Welch, et al., Int. J. Cancer 47: 227-237 (1991)), which were established from melanoma metastases, had a more than 1000-fold higher level of TF expression. When cell lysates of M24met and C8161 were electrophoretically separated and analyzed by Western blotting with mono-specific antibodies to TF, a single 47 kDa protein was identified under reducing conditions, consistent with fully glycosylated and properly processed human TF (Morrissey, et al., Thromb. Res. 50: 481-493 (1988); data not shown). High level expression of TF thus characterizes these metastatic melanoma cells.

Indirect immunofluorescence was used to assess whether TF is expressed on the tumor cell surface or is sequestered in intracellular compartments where it would likely be of minor functional significance in vivo. Specific immunoreactivity of mAbs TF8-5G9 and TF9-10H10 is demonstrated by flow cytometry using non-permeabilized M24met or C8161 cells (see Fig. 2).

Figures 2A and B illustrate the surface expression of TF on metastatic melanoma cell lines. In each instance, the number of cells is plotted on the vertical axis, while fluorescence intensity is shown on the horizontal axes. Indirect immunofluorescence and flow cytometry were used to demonstrate TF antigen on the surface of M24met (Fig. 2A) and C8161 (Fig. 2B) melanoma cell lines. Cells were incubated with 50 μ g/ml isotype matched control Ab (TIB115; solid line), or anti-TF mAb TF8-5G9 (bold line) and TF9-10H10 (dotted line), respectively. Bound mAb was visualized by a secondary goat anti-mouse IgG conjugated to fluorescein isothiocyanate. Results of representative experiments are shown in each of 2A and 2B.

Additional anti-TF mAbs to non-overlapping

epitopes provided similar results (data not shown).
The cell surface expression of TF on M24met cells was
approximately 3.5- to 4-fold lower than on C8161 cells,
consistent with the relative functional TF activities
5 of these cell lines determined in the coagulation
assay. Cell surface expression on M24met cells was
also quantified by radioligand binding analysis with
radiolabeled mAb TF8-5G9. Scatchard analysis
demonstrated saturable binding to $64,000 \pm 28,000$ sites
10 per cell with a dissociation constant of 197 ± 5 pM
(mean \pm standard deviation, $n=3$). These data are
consistent with predominant cell surface expression of
TF on the cells. TF therefore represents the major
initiator of the coagulation pathways on the surface of
15 these metastatic melanoma cells.

Example IV

Specific Inhibition of Human TF in Murine Blood

To explore whether the presence of functional TF
on the melanoma cell surface contributes to metastatic
20 tumor dissemination, a xenograft model in which human
melanoma cells are injected intravenously into mice
with severe combined immune deficiency (scid) was
chosen. Species compatibility was assessed with
recombinant human TF reconstituted into phospholipid
25 vesicles.

Figure 3A illustrates functional activity of human
TF in human and mouse plasma. Clotting time in seconds
is plotted on the vertical axis, whereas the amount of
human TF (in ng/ml) is shown on the horizontal axis.
30 The ability of phospholipid reconstituted recombinant
human TF to initiate coagulation in mouse plasma (open
circles) was evaluated in a standard clotting assay in
comparison to pooled normal human plasma (closed
circles).

35 Figure 3B shows that, in either case, a

preincubation with 30 μ g/ml TF8-5G9 inhibited TF-induced coagulation, whereas preincubation with TF9-10H10 did not. In Fig. 3B, procoagulant activity (mU/ml) is shown on the vertical axis. On the horizontal axis, results of preincubation of human vs. mouse plasma with 30 μ g/ml control (TIB115), 10H10 (TF9-10H10) or 5G9 (TF8-5G9) mAb is shown.

Human TF triggered coagulation with the same efficiency in both human and mouse plasma (Fig. 3), demonstrating that the xenograft model adequately reflects the interaction of tumor cell TF with the host's blood. Based on analysis in a purely human system, the monoclonal antibodies TF8-5G9 and TF9-10H10 have distinct functional characteristics, although they are directed to partially overlapping epitopes (Ruf, et al., Biochem. J. 278: 729-733 (1991)). Whereas mAb TF9-10H10 does not inhibit binding of factor VIIa and function of the TF-VIIa complex, mAb TF8-5G9 is a potent and rapid inhibitor of human TF in human plasma due to its ability to compete with substrate access to the TF-VIIa complex. In addition, this mAb does not inhibit TF from various non-human primates (except chimpanzee) or any lower mammal species tested to date (Morrissey, et al., Thromb. Res. 52: 247-261 (1988)), thus providing a highly-specific tool to selectively block human TF function in the xenograft model. Both the inhibitory mAb TF8-5G9 and the non-inhibitory mAb TF9-10H10 had identical functional characteristics whether in human or mouse plasma (Fig. 3). The use of the human TF-specific mAb TF8-5G9 thus allows selective inhibition of melanoma cell TF function without interfering with murine TF expressed on host cells.

Example VMelanoma Cell TF Function is Required
for Hematogenous Metastasis

Intravenous injection of M24met metastatic
5 melanoma cells into scid mice reliably produces a large
number of metastases in the lungs. Table 2 illustrates
the effect of functional inhibition of TF, in an
experimental pulmonary metastasis model. In that
table, the number of metastases per mouse in each
10 experiment is plotted against the total dose of
monoclonal antibody (or buffer solution only)
administered to each subject.

As illustrated in Table 2, M24met or C8161 cell
suspensions were incubated in buffer with or without
15 1mg of mAb TF8-5G9 mAb or its Fab fragment, mAb TF9-
10H10, or isotype control mAb TIB115 (identified as
IgG, in Table 2), for 45 minutes prior to injection
into the lateral tail vein of scid mice. When a 2mg
dose was administered, mice received a second
20 intravenous injection of 1mg mAb after 24 hours. After
21 days, all animals were sacrificed, their lungs
fixed, and the number of visible tumor nodules on the
lungs was counted. The probability of no difference
(p) between buffer control and mAb-treated groups is
25 indicated based on the non-parametric Wilcoxon rank sum
test.

Table 2

EFFECT OF FUNCTIONAL INHIBITION OF TF ON
EXPERIMENTAL PULMONARY METASTASIS

Experiment 1: 1X10⁶ M24met

<u>mAb (total dose)</u>	<u>No. Metastases/Mouse (total in both lungs)</u>						<u>p</u>
none	314	353	356	375	384	402	-
TF8-5G9 (1mg)	14	16	27	42	94	153	<0.0022
TF9-10H10 (1mg)	279	293	346	363	375	377	0.309
IgG ₁ (1mg)	281	291	292	327	368	424	0.24

Experiment 2: 1 X 10⁶ M24met

<u>mAb (total dose)</u>	<u>No. Metastases/Mouse (total in both lungs)</u>						<u>p</u>
none	41	86	145	175	196	221	-
TF8-5G9 (1mg)	0	0	0	0	0	1	<0.0022
TF8-5G9 (2mg)	0	0	0	0	0	0	<0.0022
TF8-5G9 Fab (2mg)	0	2	5	6	15	16	<0.0022

Experiment 3: 5 X 10⁶ C8161

<u>mAb (total dose)</u>	<u>No. Metastases/Mouse (total in both lungs)</u>						<u>p</u>
none	20	21	37	65	88	170	-
TF8-5G9 (2mg)	0	0	0	2	3	4	<0.0022
TF9-10H10 (2mg)	2	3	15	21	56	63	0.09

The number of experimental pulmonary metastases was significantly reduced when mice were infused with M24met cells in the presence of 1mg TF8-5G9, in comparison to a control group injected with M24met cells in buffer (see Table 2). Mice receiving TF8-5G9 also had a significant reduction in metastases when compared to animals treated with an irrelevant isotype matched control mAb (TIB115) or the anti-TF mAb TF9-10H10, which binds TF with high affinity without blocking its function. In an independent experiment, the anti-metastatic effect of TF8-5G9 was reproduced. Furthermore, Fab fragments of TF8-5G9 effectively inhibited M24met metastasis. This finding further excludes F_c -mediated effects, though considered unlikely because of the lack of anti-metastatic effects of the anti-TF mAb TF9-10H10 which is of the same isotype as TF8-5G9. The inhibition by Fab fragments was slightly less effective compared to the intact mAb; this may be a result of an observed 2- to 3-fold poorer affinity of Fab fragments of TF8-5G9 (Ruf, et al., Thromb. Haemost. 66: 529-533 (1991)). Blocking of TF function by mAb or Fab fragments, but not binding of a non-inhibitory mAb to the same receptor, reduces pulmonary metastasis consistent with a requirement for TF function in hematogenous metastasis of tumor cells.

The role of TF in metastasis was further evaluated with another metastatic melanoma cell line which exhibits high levels of TF expression. Compared to buffer control, mAb TF8-5G9 significantly inhibited experimental lung metastasis of C8161 melanoma cells. (See Table 2, Experiment 3.) Similar to M24met melanoma cells, the non-inhibitory mAb TF9-10H10 did not significantly reduce melanoma metastasis. However, there was an apparent decrease in the number of pulmonary metastases when compared to the buffer

control group. This difference, which is not statistically significant, may indicate a slight effect of the 3.5- to 4-fold higher number of TF sites and consequently bound mAb molecules on the surface of C8161 compared to M24met cells which might elicit some F_c -mediated host defense mechanisms. These data demonstrate that only functional inhibition of TF results in a significant reduction of pulmonary metastasis, providing evidence that two independently established metastatic melanoma cell lines require TF function for efficient hematogenous metastasis.

Example VI

TF Function is Required for Prolonged Adherence of Melanoma Cells

The distribution of intravenously injected radiolabeled tumor cells was studied in order to assess whether initial arrest in various organs and persistence of tumor cells is influenced by blocking of TF function (Fig. 4). Figures 4A and 4B illustrate the distribution of radiolabeled M24met cells after intravenous injection into scid mice. On the vertical axis, time in minutes (4A) or hours (4B) is shown. On the horizontal axis, the percent of injected dose is illustrated, in liver, blood, and lungs. In 4A, $p = 0.018$; in 4B, $p = 0.005$.

Cells were labeled with 5- (^{125}I) -Iodo-2'-deoxyuridine for 24 hours, washed and incubated for 45 minutes with 1mg monoclonal antibody TF8-5G9 ("open" or white bars) or buffer control ("closed" or colored bars). Radiolabeled M24met cells were injected into the tail vein of scid mice. Mice were bled and sacrificed at different time points, as shown. Means and standard deviations are based on four animals per group at each time interval. The probability of no difference occurring between groups at a given time was

assessed with the Wilcoxon rank sum test.

Inhibition of TF function on M24met cells had no effect on the rapid clearance of tumor cells from the blood, when compared to the buffer control. As early
5 as 10 minutes after injection, only 1% of infused tumor cells remained per ml of blood, and radiolabeled cells were virtually absent after 24 hours in all animals. In the livers of mice treated with the anti-TF mAb TF8-5G9, more tumor cells were initially arrested in
10 comparison to the controls. This may reflect increased clearance of a small fraction of the antibody coated cells by the reticuloendothelial system of the liver. Only traces of radiolabel (< 0.25% of the total dose) were detected in spleens and kidneys at all time
15 points, with no differences in mAb-treated or untreated animals.

The majority of tumor cells rapidly localized to the lungs with no difference between the anti-TF mAb-treated and the untreated control group. The
20 radiolabel in the lungs dropped from 75-80% of total tumor cells at 10 minutes after injection to 55-60% of the tumor cells 90 minutes after injection. This indicates that the initial distribution and the selective arrest in the lung vasculature is not
25 influenced by inhibition of TF function. However, early attachment was not sufficient to dictate successful implantation. Thus, inhibition of tumor cell TF significantly reduced the number of cells retained in the lungs four hours and 24 hours following
30 tumor cell infusion. At 24 hours, the radiolabel equivalent to 170 tumor cells was present in the lungs of animals receiving inhibitory anti-TF mAb compared to 15,000 cells in controls. Functional TF on the melanoma cells thus appears to be required for
35 prolonged adherence of tumor cells at the site of

initial arrest.

Example VII

Discussion of the Results

of Examples I-VI

5 The malignant phenotype of cells typically
incorporates aberrations in growth control, as well as
their capacity to invade and metastasize. Critical
events in the metastatic process include (1) the
initial arrest and (2) stable implantation of tumor
10 cells in the microvasculature of target organs.

 Various studies on the arrest of circulating tumor
cells discriminate between an initial phase of direct
contact between tumor cells and the vascular
endothelium and a second phase characterized by fibrin
15 generation and formation of a platelet thrombus in
association with the arrested tumor cells (Crissman, et
al., Cancer Res. 48: 4065-4072 (1988)). This indicates
that proteolytic activity is rapidly generated at the
site of tumor cell arrest, marking a second phase of
20 stabilized implantation at the endothelium.

 It is demonstrated herein that generation of
proteolytic activity on the surface of metastasizing
human melanoma cell lines is triggered by TF, the major
initiating molecule of the coagulation cascades.
25 Indeed, the cellular expression of this molecule was
observed to parallel the progression of melanoma cells
to a metastatic phenotype. Whereas normal cultured
human melanocytes or a primary melanoma derived cell
line had only trace levels of functional TF, at least a
30 1000-fold higher cell surface expression was detected
on metastasizing human melanoma cells. Since all of
the cell lines were proliferating, expression of TF did
not reflect cell cycle difference, but appeared,
rather, to be the correlate of the metastatic
35 potential.

The functional role of TF expressed on melanoma cells was explored in an experimental metastasis model in scid mice. The mAb TF8-5G9, which selectively binds and functionally neutralizes human TF in the xenograft model, markedly reduced the number of pulmonary metastases of two metastatic melanoma cell lines. This was shown to result from functional inhibition of tumor cell TF rather than non-specific host defense effects based on: (1) lack of reactivity of the murine anti-human TF mAbs with host TF; (2) inhibition of metastasis by Fab fragments of mAb TF8-5G9; and (3) unaltered implantation of pulmonary metastasis when a non-inhibitory mAb was bound to the same cell surface receptor. Inhibition of TF function did not interfere with the initial arrest of the tumor cells in the lungs of animals, but rather, abolished the prolonged localization to this organ. This study thus establishes a requisite second phase in tumor cell implantation which is dependent on tumor cell TF-driven generation of coagulation serine proteases.

The initial arrest of tumor cells appeared not to be dependent on the generation of proteolytic activity. Direct interaction between tumor cells and endothelial cells through constitutively expressed and lung-specific endothelial cell adhesion molecules, such as Lu-ECAM-1 (Zhu, et al., PNAS USA 88: 9568-9572 (1991)) may be responsible for this early attachment. This initial arrest, though necessary for the metastatic process (Zhu, et al., J. Clin. Invest. 89: 1718-1724 (1992)), is not sufficient to establish metastases. In a second phase, TF on the tumor cell surface is required to activate the coagulation cascades which, among other serine proteases, ultimately generate thrombin. Inhibition of this protease in the early phase of tumor seeding has indeed been shown to reduce

pulmonary metastasis (Esumi, et al. (Cancer Res. 51: 4549-4556 (1991))). However, the metastatic process may be dependent on various biological effects of thrombin which are mediated through proteolytic activation of the thrombin receptor (Coughlin, et al., J. Clin. Invest. 89: 351-355 (1992)).

Thrombin may trigger critical events in the early phase of tumor cell implantation. Stimulation of endothelial cells by thrombin results in rapid expression of P-selectin (Pober, et al., Transplantation 50: 537-544 (1990)) for which ligands are found on a variety of tumor cells (Aruffo, et al., PNAS USA 89: 2292-2296 (1992)). More delayed expression of E-selectin upon stimulation of endothelial cells may contribute to additional cooperative adhesive interactions through carbohydrate ligands associated with the melanoma cells (Burrows, et al., Cancer Res. 51: 4768-4775 (1991)). The avidity of adhesive receptors is modulated by signaling molecules such as platelet activating factor (PAF) which is synthesized by endothelial cells upon thrombin stimulation (Zimmerman, Immunology Today 13: 93-100 (1992)). Melanoma-endothelial cell interaction in metastasis may recapitulate the principles of leukocyte-endothelial cell interactions in inflammation (Butcher, Cell 67: 1033-1036 (1991)). This process is characterized by an initial, transient and reversible adhesion mediated by selectins, followed by more stable adherence resulting from the up-regulation and functional participation of leukocyte integrins and their counter-receptors on the stimulated endothelial cell.

Additional events may be triggered by the initial generation of proteolytic activity at the site of tumor cell arrest. Fibrin generation may protect arrested

cells from host defensive mechanisms (Gunji, et al., Cancer Res. 48: 5216-5221 (1988)). Fibrin which is deposited locally serves as a matrix for the interaction of tumor cells through their adhesive receptors which may modify or induce the expression of genes relevant for invasion and tumor growth (Hynes, Cell 69: 11-25 (1992)). Thrombin may support invasion of tumor cells by degrading the subendothelial matrix either directly (Liotta, et al., Cancer Res. 41: 4629-4636 (1981)) or indirectly by inactivation of inhibitors for other tumor cell-associated proteases (Ehrlich, et al., J. Cell. Biol. 115: 1773-1781 (1991)). Proteolytic activation of the thrombin receptor (Coughlin, et al., J. Clin. Invest. 89: 351-355 (1992)) on tumor cells or host cells may modify their cellular responses and stimulate proliferation. Platelets are deposited upon thrombin stimulation at the sites of tumor cell arrest and the inhibition of platelet function results in inhibition of metastasis in several experimental models (Karparkin, et al., J. Clin. Invest. 81: 1012-1019 (1988); Okoshi, et al., Cancer Res. 51: 6019-6024 (1991)). However, it is not known whether the stabilization of the initial fibrin clot (Weiss, et al., FASEB J. 2: 12-21 (1988)) or the release of pleiotropic growth modulators such as platelet-derived growth factor (PDGF), platelet factor 4 (PF4) or transforming growth factor beta (TGF- β) (Rusciano, et al., BioEssays 14: 185-194 (1992)) may contribute to prolonged tumor cell survival and growth. Thus, locally-generated proteases may influence additional events following the secondary stabilized localization of tumor cells.

It is not known whether occupancy of TF by ligand (factor VII or VIIa) provides additional signals to the tumor cell that favors survival and proliferation.

Binding of mAb TF8-5G9 to TF not only rapidly abolishes function, but also decreases the binding of ligand (Ruf, et al., Thromb. Haemost. 66: 529-533 (1991)). The cytoplasmic domain of TF has been found to be phosphorylated by a protein kinase C-dependent mechanism, indicating that TF is associated with other molecules involved in signal transduction (Zioncheck, et al., J. Biol. Chem. 267: 3561-3564 (1992)). In analogy to other members of the cytokine receptor family (Fuh, et al., Science 256: 1677-1680 (1992)), ligand-induced dimerization of TF may provide adaptive or proliferative signals that support, or may even be required for, the metastatic process. The findings disclosed herein establish a requirement for ligand binding and function of TF expressed on the tumor cell surface. Specific functional inhibition of this receptor molecule markedly reduces pulmonary metastasis, providing evidence that locally generated proteolytic activity at the site of tumor cell arrest is a key event in metastatic implantation.

Example VIII

Functional Inhibition of TF and Metastasis:

Dosages and Inhibitory Mechanisms

For the following experiments, the protocol described in Example V above was followed. As noted above, intravenous injection of M24met metastatic melanoma cells into scid mice reliably produces a large number of metastases in the lungs.

Tables 3A and 3B illustrate the effect of functional inhibition of TF, in an experimental pulmonary metastasis model. In the tables, the number of metastases per mouse in each experiment is plotted against the total dose of monoclonal antibody (or buffer solution only) administered to each subject.

As illustrated in Table 3A below, a dose titration

of TF8-5G9 demonstrates efficient inhibition of metastasis at lower doses, which are all capable inhibiting tissue factor function by more than 98%. This suggests that antibodies capable of inhibiting
5 tissue factor function are useful inhibitors of metastasis over a rather wide dosage range.

Table 3B depicts data from an experiment in which antibody TF8-5G9 is compared with antibody TF9-6B4, which inhibits tissue factor function via a different
10 means than TF8-5G9, through binding to a distinct epitope in the amino-terminal module of tissue factor. According to the data shown, TF9-6B4 inhibits metastasis, though perhaps somewhat less potently than
15 TF8-5G9. This difference seems to be is consistent with the reduced inhibitory potency of TF9-6B4 versus TF8-5G9 observed in plasma.

Table 3A

FUNCTIONAL INHIBITION OF TF PREVENTS
EXPERIMENTAL PULMONARY METASTASIS
- ANTIBODY DOSAGE EXPERIMENTS -

Number of Cells Injected Into Each Mouse: 0.5X10⁶ M24met

<u>mAb</u> <u>(total dose)</u>	<u>No. Metastases/Mouse (total in both lungs)</u>			
none	153	152	39	0
TF8-5G9 (1mg)	2	1	0	0
TF8-5G9 (0.5mg)	1	0	0	0
TF8-5G9 (0.1mg)	1	0	0	0
TF8-5G9 (0.05mg)	2	0	0	0

TABLE 3B

FUNCTIONAL INHIBITION OF TF PREVENTS
EXPERIMENTAL PULMONARY METASTASIS
- INHIBITORY MECHANISM -

Number of Cells Injected Into Each Mouse: 0.5 X 10⁶ M24met

<u>mAb</u> <u>(total dose)</u>	<u>No. Metastases/Mouse (total in both lungs)</u>					
none	17	19	29	51	63	75
TF8-5G9 (1mg)	0	0	0	0	0	1
TF9-6B4 (1mg)	2	4	6	7	8	9

Example IX

Inhibition of Tumor-Associated TF In Vivo

1mg of mAb TF8-5G9 was injected into the tail veins of scid mice and plasma was obtained from cardiac puncture after four hours. The inhibition of TF function by the mAb in the mouse plasma was determined by preincubation of relipidated human TF with the plasma for 30 minutes followed by determination of residual TF function. A 1:2500 dilution of the mouse plasma inhibited TF function by more than 90%. Thus, specific functional inhibition by TF8-5G9 is not lost in vivo.

In vivo inhibition of TF function in primary tumors and metastases of the melanoma cell line M24met was also analyzed, as follows. A cell suspension was implanted subcutaneously leading to subcutaneous tumor formation from which lymph node metastases developed. Tumor tissue was isolated and stored frozen. After mechanical disruption of the tumor integrity, TF functional activity was established (see Fig. 5). TF procoagulant activity was normalized on the basis of the wet weight of tumor tissue or the M24met cell pellet from culture in vitro and the quantitative differences may relate to the relative amount of tumor cells compared to host tissue.

Figure 5 illustrates the ability of anti-TF monoclonal antibodies to inhibit tumor associated TF in vivo and the TF expression of M24met melanoma cells in vivo. Tissue factor procoagulant activity in mU/mg is indicated on the vertical axis, whereas TF expression in the presence or absence of mAb TF8-5G9 in cultured cells, subcutaneous tumors, and lymph node metastases is represented by the dark bars (- TF8-5G9) and light bars (+ TF8-5G9) extending vertically from the horizontal axis.

Monoclonal antibody TF8-5G9 efficiently inhibited TF in both the subcutaneous tumor and the lymph node metastases. Residual procoagulant activity in the metastasis originates most likely from host-derived
5 activated macrophages which express TF during the immune response. Host TF is not, however, neutralized by TF8-5G9.

The foregoing specification, including the
10 specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.

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WE CLAIM:

1. A therapeutic composition comprising, in unit dose form, immunoglobulin molecules capable of binding to and inhibiting the function of human tissue factor (huTF), in a pharmaceutically acceptable excipient.
2. The composition of claim 1, wherein said molecules comprise antibodies or immunologically active fragments thereof.
3. The composition of claim 2, wherein said antibodies are capable of immunoreacting with huTF locus I or locus II epitopes, or both.
4. The composition of claim 2, wherein said antibodies are monoclonal.
5. The composition of claim 4, wherein said monoclonal antibodies are selected from the group consisting of TF8-5G9 and TF9-6B4.
6. A method of making a medicament useful for treating or inhibiting metastasis, comprising admixing a therapeutically effective amount of immunoglobulin molecules capable of binding to and inhibiting the function of cell surface-expressed huTF with a pharmaceutically acceptable carrier or excipient.
7. The method of claim 6, wherein said immunoglobulin molecules comprise antibodies or immunologically active fragments thereof.
8. The method of claim 7, wherein said antibodies are monoclonal.
9. The method of claim 8, wherein said antibodies are selected from the group consisting of TF8-5G9 and TF9-6B4.
10. The method of claim 6, wherein said medicament is formulated for intravenous administration.

11. The method of claim 6, wherein said therapeutically effective amount comprises an amount sufficient to reduce the cell surface expression of huTF by at least 20%.

5 12. The method of claim 6, wherein said therapeutically effective amount comprises an amount sufficient to produce plasma levels of said immunoglobulin molecules of about 0.05 μ g/ml to about 25 μ g/ml.

10 13. A method of identifying molecules capable of inhibiting metastasis, comprising:

(a) generating a suspension from a TF-containing source;

15 (b) incubating said suspension with a sample of said molecules for a predetermined period of time, to form an admixture; and

(c) assaying said admixture for TF specific activity, thereby determining the ability of said molecules to inhibit metastasis.

20 14. A method of identifying compounds or molecules capable of inhibiting the expression of human tissue factor (huTF) on the cell surface, comprising:

(a) culturing tumor cells;

25 (b) admixing said tumor cells with a non-cytotoxic test compound;

(c) incubating the resulting admixture for a predetermined period of time; and

(d) determining the amount of TF expressed per cell.

30 15. A method of inhibiting metastasis, comprising the administration of a therapeutically effective amount of immunoglobulin molecules capable of binding to and inhibiting the function of cell surface-expressed huTF.

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16. The method of claim 15, wherein said molecules comprise antibodies or immunologically active fragments thereof.

5 17. The method of claim 16, wherein said antibodies are monoclonal.

18. The method of claim 17, wherein said antibodies are selected from the group consisting of TF8-5G9 and TF9-6B4.

10 19. The method of claim 15, wherein said therapeutically effective amount comprises an amount sufficient to reduce the cell surface expression of huTF by at least 20%.

15 20. A method of disrupting huTF·VIIa complex formation and function comprising the administration of an effective amount of antibodies or immunologically active fragments thereof, wherein said antibodies or fragments are capable of binding to cell surface-expressed huTF.

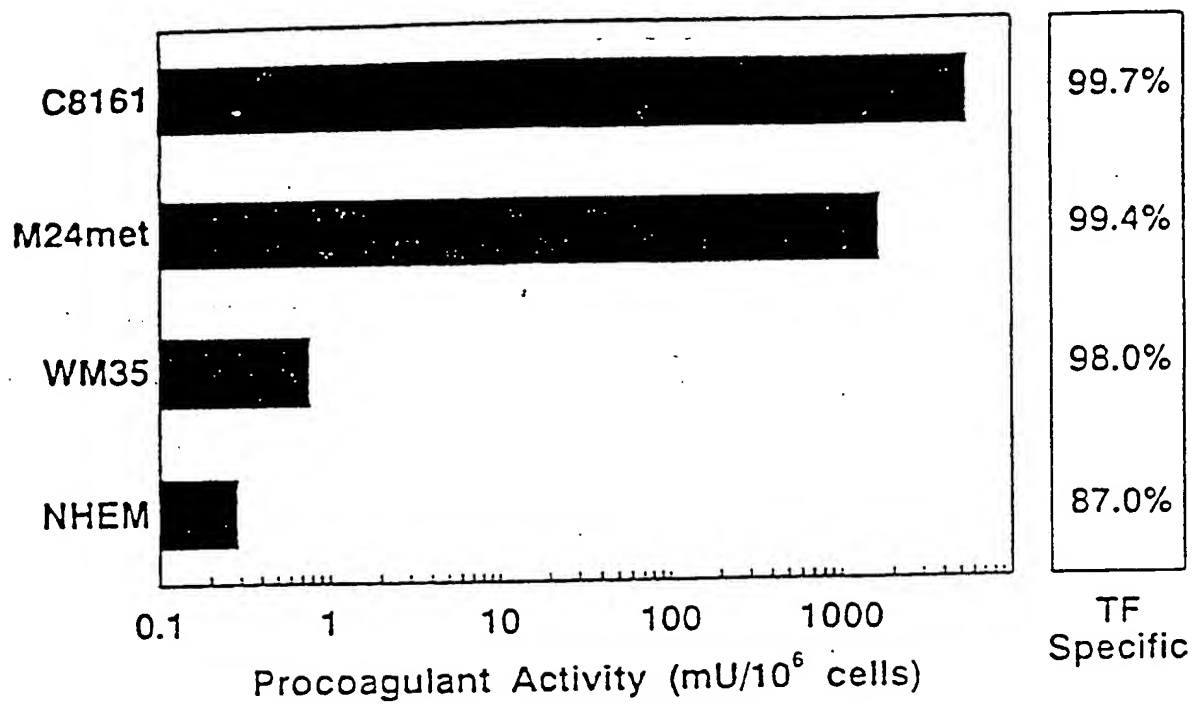


Fig. 1

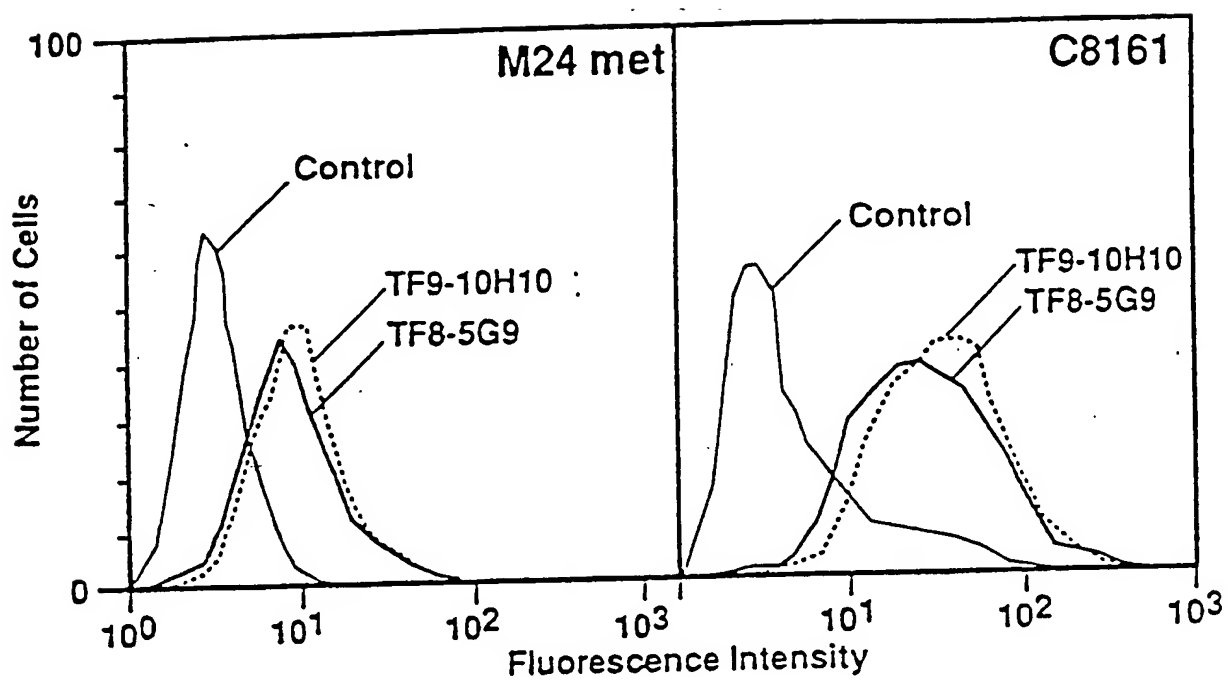


Fig. 2A

Fig. 2B

Fig. 2

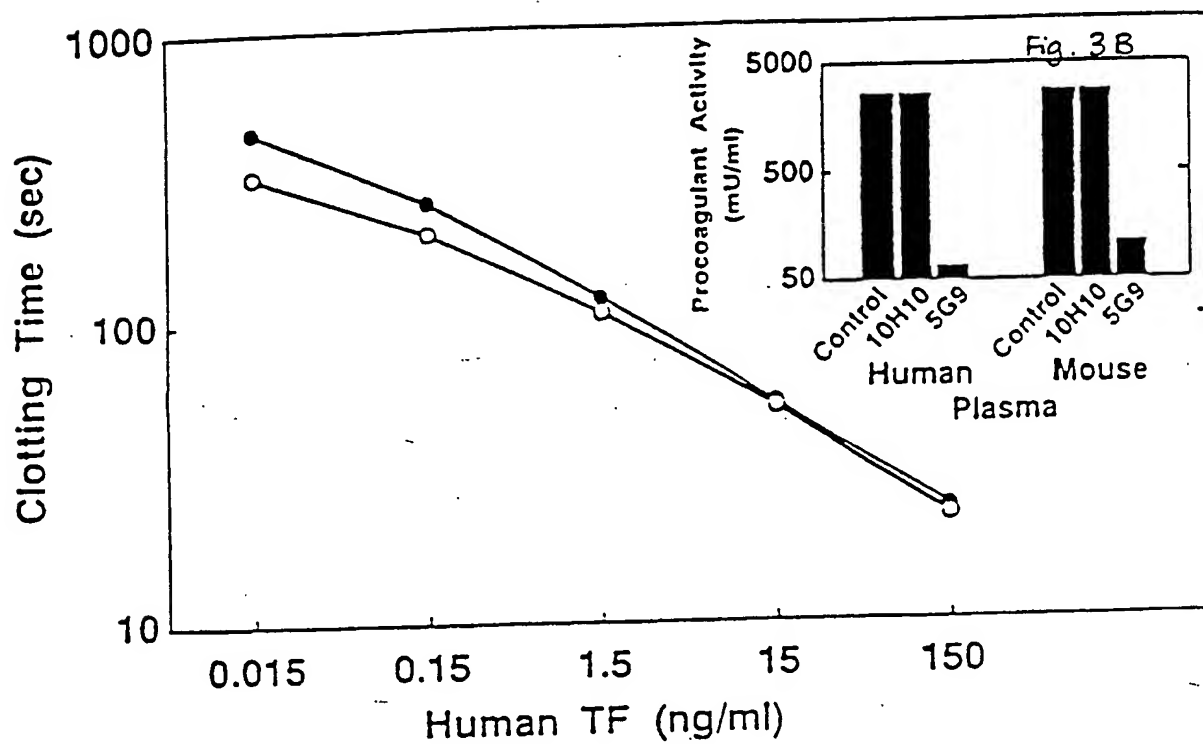
Fig.
3A

Fig. 3

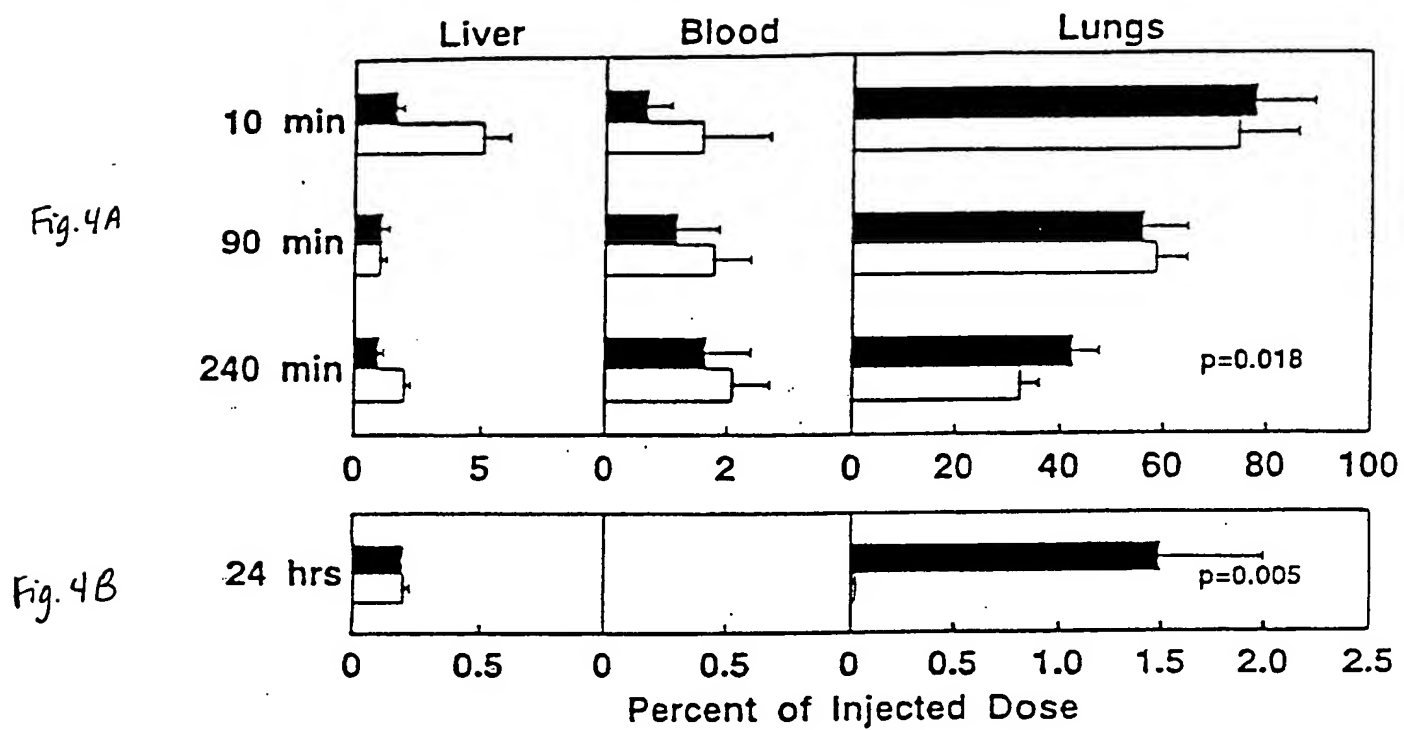


Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Thrombosis and Haemostasis, Volume 66, issued November 1991, W. Ruf et al., "An Anti-Tissue Factor Monoclonal Antibody Which Inhibits TF-VIIa Complex Is a Potent Anticoagulant in Plasma", pages 529-533, see entire document.	1-20
Y	Cancer Research, Volume 49, issued 01 October 1989, J. M. Silberberg et al., "Identification of Tissue Factor in Two Human Pancreatic Cancer Cell Lines", pages 5443-5447, see entire document.	1-20
Y	J. Cell. Biol., Volume 109, issued July 1989, T.A. Drake et al., "Functional Tissue Factor Is Entirely Cell Surface Expressed on Lipopolysaccharide-Stimulated Human Blood Monocytes and a Constitutively Tissue Factor-Producing Neoplastic Cell Line", pages 389-395, see entire document.	1-20
Y	Cancer Research, Volume 46, issued December 1986, M.B. Donati et al., "Cancer Procoagulant in Human Tumor Cells: Evidence from Melanoma Patients", pages 6471-6474, see entire document.	1-20
Y	Thrombosis Research, No. 3, Volume 52, issued December 1988, J.H. Morrissey et al., "Monoclonal Antibody Analysis of Purified and Cell-Associated Tissue Factor", pages 247-261, see entire document.	1-20
Y	Thrombosis Research, Volume 50, No. 4, issued May 1988, J.H. Morrissey et al., "Resolution of Monomeric and Heterodimeric Forms of Tissue Factor, The High-Affinity Cellular Receptor for Factor VII", pages 481-493, see entire document.	1-20

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INHIBITION OF TUMOR METASTASIS VIA NEUTRALIZATION OF TISSUE FACTOR
FUNCTION (English)

Patent Assignee: SCRIPPS RESEARCH INST

Author (Inventor): RUF WOLFRAM; MUELLER BARBARA M; REISFELD RALPH A;
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Author (Inventor): RUF WOLFRAM (US); MUELLER BARBARA M (US);
REISFELD RALPH A (US); EDGINGTON THOMAS S (US)

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COUNTRIES FOR REGIONAL PATENTS CITED IN A
PUBLISHED APPL. WITH SEARCH REPORT)
AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT
SE

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WO 9405328	P	19960828	WO 122	EP: PCT APP. NOT ENT. EUROP. PHASE (EP: PCT ANM. NICHT IN EUROP. PHASE EING.)